U.PORTO

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Investigation of the functional role of osteopontin and its alternatively spliced variants in thyroid carcinoma

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Lista de Publicações / List of Publications

Ao abrigo do Art. 8º do Decreto-Lei nº 388/70, fazem parte integrante desta Dissertação os seguintes trabalhos já publicados, ou submetidos para publicação:

Paper I – Ferreira LB, Tavares C, Pestana A, Leite C, Eloy C, Pinto MT, Castro P, Batista R, Rios E, Sobrinho-Simões M, Gimba ERP, Soares P. Osteopontin-a splice variant is overexpressed in papillary thyroid carcinoma and modulates invasive behavior. OncoTarget, 2016.

Paper II – Ferreira LB, Lima R, Andreia, Eloy C, Pestana A, Tavares C, Sobrinho-Simões M, Gimba ERP, Soares P. OPNa expression is associated with the formation of psammoma bodies in papillary thyroid cancer: *in vivo* and *in vitro* evidences. In preparation.

Paper III – Ferreira LB, Eloy C, Pestana A, Lyra J, Moura M, Prazeres H, Tavares C, Sobrinho-Simões M, Gimba ERP, Soares P. Osteopontin expression is correlated with differentiation and good prognosis in medullary thyroid carcinoma. European Journal of Endocrinology, v. 174, p. EJE-15-0577, 2016.

Os seguintes artigos publicados durante o curso de doutoramento, não fazem parte do corpo de resultados da tese, mas parte do seu conteúdo foi usado na sua introdução e discussão

Appendix I – Eloy C, Ferreira LB, Salgado C, Soares P, Sobrinho-Simões M. Poorly differentiated and undifferentiated thyroid carcinomas. Turkish Journal of Pathology, v. 31, p. 48, 2015.

Appendix II – Tilli T, Ferreira LB, Gimba ERP. Osteopontin-c mediates the upregulation of androgen responsive genes in LNCaP cells through PI3K/Akt and androgen receptor signaling. Oncology Letters, v. 9, p. 3, 2015.

Em cumprimento com o disposto no Decreto-Lei nº 388/70, declara que participou ativamente na recolha e estudo do material incluído em todos os trabalhos, redigiu os artigos I, II, III e parte do Apêndice I, e colaborou na Discussão do Apêndice II.

Os Enigmas

Perguntastes-me o que fia o crustáceo entre as suas patas de ouro
e eu vos respondo: O mar é que sabe.
Dizeis-me o que espera a caravela no seu sino transparente? O que espera?
Eu vos digo, espera como vós o tempo.
Perguntais-me a quem atinge o braço da alga Macrocustis?
Indagai-o, a certa hora, em certo mar que conheço.
Sem dúvida perguntar-me-eis pelo marfim maldito do narval, para que eu vos responda de que modo
o unicórnio marinho agoniza arpoado.

Perguntais-me talvez pelas penas alcionárias que tremem
nas puras origens da maré austral?
E sobre a construção cristalina do pólipo enredastes, sem dúvida,
uma pergunta mais, desfiando-a agora?
Quereis saber a elétrica matéria dos ouriços do fundo?
A armada estalactite que caminha a quebrar-se?
O anzol do peixe pescador, a música estendida na profundidade, como um fio na água?

Eu vos quero dizer que isso quem sabe é o mar, que a vida em suas arcas
é vasta como a areia, inumerável e pura
e entre as uvas sanguinárias o tempo poliu
a dureza de uma pétala, a luz da medusa
e debulhou o ramo de suas fibras corais
de uma cornucópia de nácar infinito.

Eu não sou senão a rede vazia que adianta
os olhos humanos, mortos naquelas trevas,
acostumados ao triângulo, medidas
de um tímido hemisfério da laranja.

Andei como vós, escarvando
a estrela interminável,
e na minha rede, na noite, acordei nu,
única presa, peixe encerrado no vento.

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List of Abbreviations

$\alpha v\beta 3$ – integrin binding motif

131I – iodine 131

AKT1 – v-akt murine thymoma viral oncogene homolog 1

ATA – american thyroid association

BRAF – b-raf oncogene

BSP - bone sialoprotein

BSP 1 - bone sialoprotein 1

BSP 2 - bone sialoprotein 2

CCH – c-cell hyperplasia

CD44 – cluster differentiation 44

$CD68^{+}$ – cluster differentiation 68 positive

cGMP – cyclic guanosine monophosphate

cPTC - classic variant of PTC

CT - computed tomography

CTNNB1 – catenin beta 1

EBRT - external beam radiation therapy

ECM – extracellular matrix

EGF - epidermal growth factor

EMT - epithelial-mesenchymal transition

eOPN – extracellular OPN

Eta-1 - early T- lymphocyte activation gene I

FNA - fine-needle aspiration

FTC - follicular thyroid carcinoma

fvPTC – follicular variant of PTC

HCC - hepatocellular carcinoma

HCC - hürthle cell carcinoma

iOPN- intracellular OPN

MEN2 – multiple endocrine neoplasia type 2

MMPs – metalloproteinases

MTC - medullary thyroid carcinoma

OPN – osteopontin

OPN-SV – splice variants of osteopontin

PAX8 - paired box gene 8

PB – psammoma bodies

PCCl3 - rat thyroid follicular cell line

PDTC - poorly differentiated thyroid carcinoma

PI3K-AKT – phosphoinositide 3-kinase/protein kinase B

PIK3CA – phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha

PPARγ - peroxisome proliferator- activated receptor γ

pre-mRNA – precursor mRNA

PTC - papillary thyroid carcinoma

PTEN – phosphatase and tensin homolog

PTM – post translational modifications

RAI - radioactive iodine

RAS – retrovirus associated sequence oncogene

RET – rearranged during transfection oncogene

RET-PTC – rearrangement of the RET oncogene

RGD - tripeptide Arg-Gly-Asp

Ser – serine amino-acid

SIBLING - small integrin-binding ligand n-linked glycoproteins

SPP1 - secreted phosphoprotein 1

STRN/ALK – striatin/anaplastic lymphoma kinase fusion

SV – splice variants

SVVYGLR - serine–valine–valine–tyrosine–glutamate–leucine–arginine domain

T3 - triiodothyronine

T4 - thyroxine

TC- thyroid cancer

TERT - telomerase reverse transcriptase

Tg - thyroglobulin

TGF-b - transforming growth factor-beta

Thr – threonine amino-acid

TME - tumour microenvironment

TP53 – tumour suppressor protein 53

TPO - thyroperoxidase

TRH - thyrotropin-releasing hormone

TSH - thyroid stimulating hormone

UTC - undifferentiated thyroid carcinoma

VEGF – vascular endothelial growth factor

WDTC - well-differentiated thyroid carcinoma

WHO - world health organization

Abstract

Osteopontin (OPN) has been reported as one of the most overexpressed molecules in PTC. OPN is a secreted extracellular matrix (ECM) protein that is encoded by the highly conserved *SPP1* gene; its expression levels were reported to be increased in several types of human cancers, such as prostate carcinoma, melanoma, and ovary, lung and gastric carcinoma. OPN primary transcript is subjected to alternative splicing, generating three OPN splicing variants (OPN-SV): the full-length OPNa and the shorter variants OPNb and OPNc (lacking exons 5 and 4, respectively). The three OPN-SV have been shown to play specific roles in different types of tumours and, to date, their functions in TC remain to be determined. Thyroid cancer (TC) is the most prevalent endocrine malignancy, and the fifth most frequent cancer in women. Thyroid is composed by follicular cells and by parafollicular or C-cells. The majority of TCs are derived from follicular cells, being differentiated thyroid cancer (DTC), comprising papillary thyroid cancer (PTC) and follicular thyroid cancer (FTC), the most common subtypes and accounting for 90–95% of all cases. Parafollicular cells give rise to medullary thyroid cancer (MTC). TCs frequently present aberrant expression of different gene products that may be related to cancer progression.

In the first part we analysed the expression profile of total OPN and OPN-SV in TC derived from follicular cells, the correlation of such expression with clinicopathological and molecular features, and the functional roles of OPN-SV in follicular tumour-derived cells. We showed that tOPN and OPNa are overexpressed in classic papillary thyroid carcinoma (cPTC) in comparison to adjacent thyroid, adenoma and follicular variant of papillary thyroid carcinoma (fvPTC) tissues. In cPTC, OPNa overexpression is associated with larger tumour size, vascular invasion, extrathyroid extension and $BRAF^{V600E}$ mutation. We found that TC cell lines overexpressing OPNa exhibited increased proliferation, migration, motility and *in vivo* invasion. Conditioned medium secreted from cells overexpressing OPNa induce MMP2 and MMP9 metalloproteinases activity. Taking together all the data we suggested that OPNa serve as a cancer promoter factor in follicular cell derived via the regulation of MMPs activity.

In the second part we explored the contributions of OPN in the formation of psammoma bodies (PB). PB are concentric lamellated calcified structures, observed almost exclusively in cPTCs (about 50% of all PTC cases), their clinicopathological significance remains unclarified. The presence of PB in PTC has been associated with younger patients and lymph node metastasis. We found that overexpression of OPNa was significantly associated with presence of PB in PTC samples. Fitting with these results we observed furthermore that OPNa overexpression promotes calcification and substantial collagen synthesis in thyroid cancer cell lines.

In the last part of the thesis we focused on exploring the expression profile of total OPN (all variants) and OPN-SV in MTC (tumours derived from parafollicular cells) and we evaluated if OPN expression was associated with clinicopathological data and we also assessed the role of OPN-SV in a parafollicular- tumour derived cell line (TT). We observed that OPN is expressed in MTCs and that such expression is associated with smaller tumour size, less invasive features and overexpression of nuclear PTEN. We reported for the first time the OPN expression in the specialised neuroendocrine cells of the thyroid, and showed that such expression was more marked in normal C-cell and hyperplastic C-cells that in MTCs thus supporting this assumption that OPNa may play a role in C-cell differentiation. In accordance with the data obtained in the clinicopathological study we observed that TT cells overexpressing OPNa variant show reduced proliferation and viability, suggesting that OPN may contribute to prevent tumour progression.

In conclusion, in this thesis we explore some of the roles that the matricellular protein OPN can have in endocrine and neuroendocrine thyroid cells and their respective tumours. We show that OPNa is the most prominent OPN variant in both endocrine and neuroendocrine tumour models. We also show that OPNa appears to play opposite role in the two models: in parafollicular-cell derived tumours OPNa acts as a differentiation marker whereas in follicular-cell derived tumours OPNa acts as a contributor for cancer progression. We also show that OPNa may have a strong effect in the calcification process in PTCs, participating on PB formation. Summing of, our findings highlight OPNa variant as a major TC biomarker, and provide an interesting basis for exploring several possibilities with regard to the etiopathogenesis and therapy of the

two main types of TC. From a practical standpoint our findings support the assumption that OPNa may serve as a therapy target in PTC.

Resumo

A OPN é uma proteína da matriz extracelular segregada por vários tipos celulares e codificada por um gene altamente conservado (*SPP1*); os seus níveis de expressão estão aumentados em vários tipos de cancros humanos, tais como os carcinomas da próstata, ovário, pulmão e estomago e o melanoma. O transcrito primário da OPN é submetido a *splicing* alternativo, gerando três variantes da OPN (OPN-SV): OPNa que corresponde à variante completa, e as variantes mais curtas OPNb e OPNc que faltam os exãos 5 e 4, respetivamente. O carcinoma da tireoide (CT) é a neoplasia maligna endócrina mais prevalente, e o quinto cancro mais frequente nas mulheres. A tireoide é composta por células parafoliculares, ou células C, e por células foliculares. A maioria dos CTs são derivados das células foliculares, sendo o carcinoma diferenciado da tireoide (CDT), que compreende o carcinoma papilar (CPT) e o carcinoma folicular (CFT), os subtipos mais comuns correspondendo a 90-95% de todos os casos. As células parafoliculares dão origem ao carcinoma medular da tireoide (CMT). CTs apresentam frequentemente expressão aberrante –normalmente sobre-expressão- de produtos de genes diferentes, havendo evidencia de que essas alterações de expressão podem estar relacionadas com a progressão dos cancros. A osteopontina (OPN) foi apontada como uma das moléculas mais sobre-expressas em CPT. Vários estudos demonstraram que as três OPN-SV podem desempenhar funções específicas em diferentes tipos de tumores, mas eram desconhecidas as suas funções em CTs quando esta Tese foi iniciada.

Na primeira parte desta Tese, analisámos o perfil de expressão da OPN total e das OPN-SV em CT derivados de células foliculares, a correlação dessa expressão com características clínicas e moleculares, e os papéis funcionais das OPN-SV em linhas celulares de origem folicular. Mostrámos que a OPN total e OPNa estão sobre-expressas nas formas convencionais do carcinoma papilar da tireoide (cCPT) em comparação com a tireoide adjacente, adenoma e variante folicular de carcinoma papilar da tireoide (fvCPT). Nos cCPTs, a sobre-expressão da OPNa está associada com tumores maiores, invasão vascular, extensão extratireoideia e mutação do $BRAF^{V600E}$. Mostrámos também que linhas celulares de CT com sobre-expressão de OPNa tinham proliferação, migração, motilidade e invasão *in vivo* substancialmente aumentadas. O meio condicionado por células de linhas que sobre-expressam a OPNa e a segregam induzem atividade aumentada das metaloproteinases MMP2 e MMP9. Em conjunto os nossos resultados

sugerem que a OPNa se comporta como um promotor neoplásico nos carcinomas derivados de células foliculares através da uma ação em MMPs.

Na segunda parte explorámos as contribuições da OPN na formação dos corpos psamomatosos (PB). Os PB são estruturas calcificadas lamelares concêntricas observadas quase exclusivamente nas formas clássicas de CPTs. Embora sejam encontrados em cerca de 50% de todos os casos de CPTs, o seu significado clínico-patológico permanece não esclarecido. A presença de PB em CPTs foi associada a doentes mais jovens e a metástases em gânglios linfáticos. Observamos que a sobre-expressão de transcritos da OPNa se associava significativamente à presença de PB em amostras de CPTs. Demonstrámos também que a OPNa tem uma forte capacidade para promover a síntese de colagénio e a calcificação em linhas celulares de carcinoma da tireoide.

Na última parte desta Tese, explorámos o perfil de expressão da OPN total (todas as variantes) e das OPN-SV em CMT avaliando as relações dessa expressão com os dados clínico-patológicos. Também avaliámos o papel das OPN-SV numa linha celular derivada de MTC (TT). Neste trabalho, demonstrámos que a OPN é expressa em CTMs e que essa expressão se associa com tumores pequenos, menos invasores e mostrando sobre-expressão nuclear do PTEN. Mostramos pela primeira vez a expressão da OPN em células neuroendócrinas especializados da tireoide (células C) e observamos que essa expressão era mais elevada em células C "normais" e células C hiperplásicas do que em MTCs. Estes achados lavaram-nos a concluir que a OPNa deverá desempenhar um papel na diferenciação das células C. Além disso, mostrámos que as células TT que sobre-expressam a variante OPNa têm proliferação e viabilidade diminuídas, sugerindo que a OPN poderá contribuir para prevenir a progressão dos MTCs.

Em conclusão, nesta Tese explorámos alguns dos diversos papéis que a proteína matricelular OPN desempenha em células de tireoide endócrinas e neuroendócrinas e nos tumores deles derivados. Mostrámos que a OPNa é a variante mais proeminente em ambos os modelos tumorais MTC e PTC. Além disso, mostrámos que OPNa desempenha aparentemente um papel oposto em carcinomas com origem em células C (MTC) e em carcinomas com origem em células foliculares (PTC e FTC). Nas células parafoliculares a OPNa serve como um marcador de diferenciação enquanto nas células foliculares a OPNa parece contribuir para a progressão das neoplasias. Finalmente, demonstrámos que a OPNa tem um forte efeito no processo de calcificação em cCPTs, participando na formação dos PB. Em resumo, os nossos resultados identificam a variante OPNa como um biomarcador de CT, e fornecem uma base interessante para explorar várias

possibilidades com relação à etiopatogenia e terapia dos dois principais tipos de CTs. De um ponto de vista prático os nossos resultados suportam a hipótese de que a OPNa pode servir como um alvo de terapia em CPT.

Chapter 1 – Introduction

1.1 Osteopontin

1.1.1 Osteopontin (OPN)

OPN was first identified and described by Senger *et al*. in 1979, as a secreted transformation-specific phosphoprotein [1]. Ten years later, OPN was rediscovered by molecular cloning of the transformation-associated gene 2ar [2]. Together with OPN, but independently, was identified a bone sialoprotein (BSP), as a major sialoprotein in bone extracellular matrix (ECM) [3-5]. Both proteins were initially named bone sialoprotein 1 (BSP 1) and bone sialoprotein 2 (BSP 2) [3]. The name osteopontin was introduced to reflect the capacity of the protein to bridge hydroxyapatite crystals through RGD and polyaspartic acid motifs discovered in the primary sequence of the protein [6]. The term "osteo" means bone and the term "pontin" is a latin word derived from "pons" that means bridge. Nonetheless, the same protein was then described as a putative lymphokine produced by activated lymphocytes and macrophages and called Eta-1 (early T- lymphocyte activation gene I) [7], and thus a more general pattern of expression for OPN was emerging. Subsequently, was introduced as an alternative name "secreted phosphoprotein" (SPP1), to reflect a broader functional role of this protein. Nevertheless, the name osteopontin has largely been retained, and was kept as the nomenclature used for the human gene [8].

Hence, OPN belongs to the SIBLING family of proteins. SIBLING (Small Integrin-Binding Ligand N-linked Glycoproteins) proteins are a family of glycoproteins that are encoded by a gene cluster on chromosomes 4 and 5 in humans and mice, respectively, and play important roles in mineralization [9].

1.1.2 Structure and post-translational modification (PTM)

OPN is expressed by a single-copy gene as a 34-kDa nascent protein composed of 300 amino acid residues. Its molecular weight is 44 to 66 KDa depending on the

species and cell types [10]. The human gene contains 7 exons, spanning 1.1kb. OPN is a high negatively charged protein, being hydrophilic and acidic in nature. It is also rich in aspartic and glutamic acid and serine, and contains a polyaspartic acid motif, through which the protein can bind to hydroxyapatite and calcium ions, and a RGD sequence which can mediate cell attachment [11], shown in Figure 1.

OPN contain distinct secondary structure elements with reduced conformational edibility and displays distinct tertiary contacts that encompass binding sites for $\alpha v\beta 3$ integrin and heparin [12]. Additionally, OPN can be cleaved by thrombin, exposing the cryptic C-terminal $\alpha 4\beta 1$ and $\alpha 9\beta 1$ integrin-binding motif (SVVYGLR) [13].

OPN is extensively post-translationally modified. It has multiple Ser and Thr phosphorylation sites, as well as sites for N- and O-linked glycosylation. Thus, a variety of phosphorylation, glycosylation, sulphation and proteolytic processing occurs. These modifications have significant effects on the structure and biological properties of the protein, generating different OPN functional variants, presenting tissue specificity and functional versatility [14, 15]. For instance, normal rat kidney cells secrete both phosphorylated and non-phosphorylated variants of OPN, which diverge in glycosylation patterns and physiological properties [16]. Phosphorylation is one of the PTMs most studied and has been shown to be very important for a diversity of functions of the protein. Therefore, Shiraga *et al*., [17] showed that the regulatory roles of OPN in normal and pathological mineralization are highly dependent on the phosphorylation status of the protein. Yet, dephosphorylation of OPN interfere with its ability to inhibit hydroxyapatite formation [18], whereas OPN phosphorylation is necessary for inhibition of calcium oxalate crystallization in urine [19] and calcification of vascular smooth muscle cells [20]. OPN has also been shown to promote trophoblastic cell migration in a process that is dependent on protein phosphorylation levels [21].

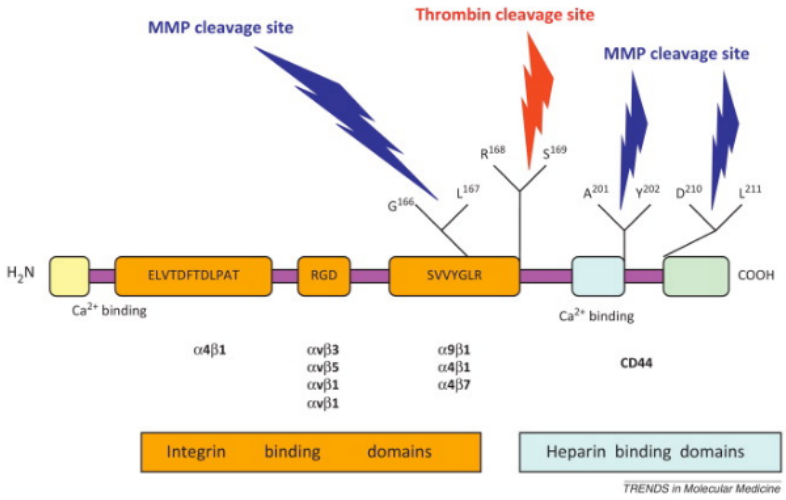

Figure 1: OPN molecular structure and interactions. OPN binds to integrins, transmembrane and dimeric proteins, consisting of alfa and beta subunits. It has several cell interacting domains that facilitate integrin binding in different cell types: an arginine–glycine–aspartic acid (RGD) cell binding sequence, which interacts with cell surface integrins αvß3, αvß1, αvß5, and α8ß1; a serine–valine–valine–tyrosine–glutamate–leucine–arginine (SVVYGLR)-containing domain, located between the RGD sequence and the thrombin cleavage site, which interacts with α9ß1, α4ß1, and α4ß7; a ELVTDFTDLPAT domain is also reported to bind to α4ß1; a calcium binding site and two heparin binding domains also exist: the heparin binding domains bind to CD44 receptor. Furthermore, OPN can be cleaved by at least two classes of proteases: thrombin and matrix metalloproteases (MMPs). *In vitro*, fragments generated by cleavage expose new active domains that may impart new activities. Adapted from: Musso G *et al*., 2013. Cell.

1.1.3 Alternative splicing

By definition alternative splicing is the process by which pre-mRNA molecules are spliced in distinct ways, and this process is important for normal development to create diversity of proteins in complex organisms [22]. Also, alternative splicing has been found to be associated with cancer and other diseases such as Frasier syndrome, Parkinson's disease, cystic fibrosis, retinitis pigmentosa, spinal muscular atrophy, and myotonic dystrophy [23, 24]. In pathological conditions may occur aberrant splicing of pre-mRNA, which can be ultimately caused by errors in RNA splicing or in its regulation. Additionally, splice variants can be produced by disruption of splicing patterns, and these splice variants (SV) can have different functions [25]. OPN is encoded by a single copy gene, although can be expressed in a number of different variants, that differ as a

result of alternative translation [26], alternative splicing [10], but also mainly in the extent of PTMs [27]. The human OPN mRNA is subjected to alternative splicing, generating three spliced variants (OPN-SV). OPNa is the full length variant, OPNb lacks exon 5 and OPNc lacks exon 4, as shown in Figure 2.

The first description of OPN-SV was reported by Saitoh Y *et al*. in 1995 [28], in glioma cells. Since this date, many other papers have been published, describing the expression patterns and the roles of OPN-SV in different cell contexts, notably in cancer cells. Tissue specific-expression and functional roles are the main features of these OPN-SV, and, nevertheless, each OPN-SV can act as a single OPN-SV or in concert, or they may even have opposite roles in the same cell type. Acting as a single OPN-SV, for instance, in ovarian and breast tumour cells, OPNc activates tumour-progression features [29, 30]. As examples of OPN-SV that acts in concert, OPNb and OPNc exerts several pro-tumorigenic roles, through activating different steps of tumour progression in prostate cancer cells [31]. Also, in human glioma cells, OPNa and OPNc activate invasion [32]. In mesothelioma cells, OPNa, OPNb and OPNc demonstrate different activities in cell proliferation, wound closure and invasion assays, being OPNa and OPNb the pro-tumorigenic variants [33]. In other tumour types, different OPN-SV can have different functions or show opposite roles, activating different aspects of tumour progression. In lung cancer cells, OPNb affect proliferation, while OPNc expression is correlated with tumour-cell invasion [34]. Other studies have demonstrated that OPNa is overexpressed in lung cancer and stimulates several tumorigenic features, whereas OPNb has fewer effects than OPNa. Even in this tumour type, OPNc inhibits proliferation and other important cancer hallmarks, such as angiogenesis and VEGF secretion [35, 36]. In hepatocellular carcinoma (HCC) cells, OPN-SV effects depend on the cell line tested. Tumour tissues predominantly express OPNa and OPNb, whereas normal liver tissues mainly express OPNc [37]. Other investigators have demonstrated that in HCC cells, increased expression of OPNc is associated with cellular invasion and appears to correlate with metastatic potential [38].

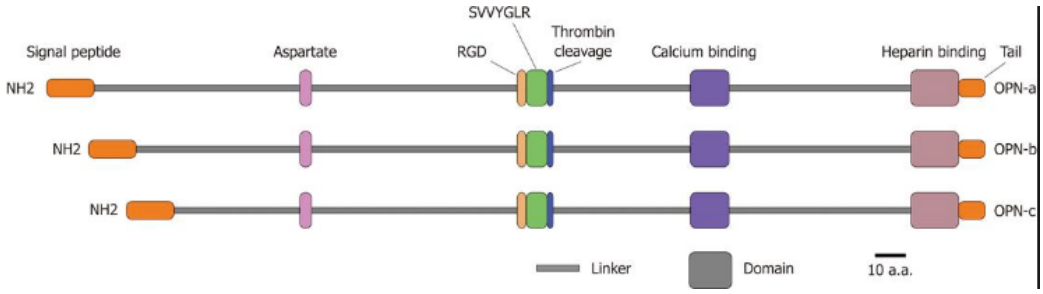

Figure 2: Structural features of OPN-SV. OPNa, OPNb and OPNc, are known. These three splice variants contain identical domains [aspartate, arginine-glycine-aspartate, Ser-Val-Val-Tyr-Gly-Leu-Arg (SVVYGLR), thrombin cleavage, calcium binding and heparin binding], which are linked together through several linkers. These variants distinguish themselves by having a variable length between signal peptide and aspartate domain linker. RGD: Arginine-glycine-aspartate. Adapted from: Cao *et al*., 2012.

1.1.4 Intra and extra-cellular OPN

Concerning location of OPN, although it has been mainly studied as a secreted or extracellular protein, some OPN molecules are not secreted, remaining inside the cells. Therefore, it has been shown that OPN can be found both in the cytoplasm and in the nucleus [26]. Many studies have demonstrated that intracellular OPN (iOPN) has biological functions distinct from extracellular OPN (eOPN). Further, it has been proposed that iOPN and eOPN can also act together [26, 39]. Mouse iOPN and eOPN variants represent alternative translational products of a single full-length OPN transcript. Translation of eOPN is initiated from the 50 canonical AUG start site, whereas translation of the iOPN variant is initiated from a downstream non-AUG codon. Downstream translation of iOPN is accompanied by deletion of the N-terminal 16-aa signal sequence, allowing the shortened protein products to remain in the cytoplasm, but not in secretory vesicles [26, 39]. However, the molecular mechanisms by which iOPN and eOPN have been generated in human cells have not been described and none iOPN SV, in any other species, has been reported. Regarding their mechanism of action, it was reported that similarly to eOPN, iOPN variants can also be associated to CD44 complex [40].

Overall, among the different variants of OPN, the extracellular human OPN-SV are the ones that have been widely studied in different cell contexts, and their role in cell physiology, notably in cancer cells, has been extensively investigated [33, 41, 42].

1.1.5 Regulation

OPN up-regulation is modulated by a diversity of factors, including hormones (steroids, retinoic acid, glucocorticosteroids and 1,25-dihydroxy vitamin D3), inflammatory cytokines and growth & differentiation factors, such as epidermal growth factor (EGF), platelet-derived growth factor and transforming growth factor-Beta (TGF-β). These factors influence gene transcriptional rates, mRNA processing, stability and protein translation, as well as PTMs maturation [43].

Additionally, OPN can be down-regulated by bisphosphonates in bone and kidney [44, 45]. Yet, OPN expression can be suppressed in vascular smooth muscle cells by cGMP-dependent protein kinase, which is a mediator of nitric oxide and cGMP signalling [46].

1.1.6 Functions

OPN is a multifunctional molecule that is involved in both physiological and pathophysiological processes. As the biological function of OPN can be regulated at both post-transcriptional and post-translational levels, OPN from various cellular sources might have diverse structural characteristics that are reflected in its various processes [47, 48], including bone remodelling; calcification (inhibition of urinary crystallization and cardiovascular calcification); immune response (macrophage cytokine production, T cell activation, stimulation of Th1 response); inflammation (both acute and chronic); regulation of cell adhesion migration; cell survival; oncogenesis and cancer progression; angiogenesis; inhibition of apoptosis; diseases such as restenosis, arterial neointimal hypeplasia, artherosclerosis and myocardial necrosis [49]. OPN has also been recognized as an important luminal regulator, due to its expression by epithelial cells covering luminal cavities capable of active secretion and absorption of nutrients [45].

Studies in OPN-knockout mice have suggested the existence of structural and functional differences between tumour- derived OPN and the OPN isoforms that are

relevant for host defences. There is evidence suggesting that tumour- derived OPN is unique (i.e. structurally different from OPN derived from untransformed cells) and lacks important domains. Thus, expression of structurally altered OPN by cancer cells can represent a mechanism of immune evasion [50].

1.1.7 Mechanisms of action

Is widely known that OPN act by binding to cell surface receptors, such as integrins and CD44 (Figure 3), being integrins the main receptors used by OPN to cell adhesion. OPN bind to different cell types by both αv (β1, β3, β5) and (α4, α8, α9) β1 integrin chains. The interaction of OPN with integrin is mediated by the RGD motif (although not limited to) and on a high integrin receptor activation state. Therefore, OPN also interacts with αvβ1 and α4β1 integrins through the sequence SVVYGLR, which is thought to be exposed upon putative thrombin cleavage, adjacent to, but distinct from, the RGD motif [51, 52]. The amino- and carboxy-terminal parts of OPN non-GRGDS (Gly-Arg-Gly-Asp-Ser) region also mediate cell attachment. The αvβ3 integrin, which can be regulated by OPN, is believed to be primarily responsible for the adhesion and migratory properties [53].

OPN can also bind intra or extracellularly to CD44 and its various isoforms, which is the main cell surface receptor for hyaluronate. CD44 is expressed in a variety of cells such as osteoclasts, osteoblast, osteocytes, epithelial cells, endothelial cells, fibroblasts and smooth muscle cells [54]. The binding of OPN to CD44 activate signalling pathways, such as PLC-g/ PKC/PI 3-kinase, leading to cell motility and survival [49].

Previous reports showed that the interaction of OPN and CD44 enhances formation of foci, invasion and tumorigenesis in H-Ras-V12-transformed cells through the Rac-mediated pathway, and these effects were abolished upon treatment with a CD44 blocking antibody [55]. The OPN and CD44 interactions can further be mediated through integrins, as there is some evidence that OPN binding by CD44 variants and β-containing integrins could cooperate to promote cell spreading and migration [56].

Regarding the activation of signalling pathways by OPN-SV, many reports have emerged describing the roles of OPN-SV on activating different aspects of tumour progression. In glioma cells, for instance, OPNa and OPNc induce cellular invasion by binding αvβ3 integrin, activating PI3K/AKT/NF-kB pathway [32]. In ovarian and prostate

cancer tumour cells, several aspects of tumour progression are activated by OPNc overexpression, also being modulated by PI3K/AKT signalling pathway [30, 31, 57]. In HCC cell lines, OPN-SV can differentially modulate its migratory activity, probably by differentially activating signalling pathways involved in tumour metastasis, such as urokinase-type plasminogen activator (uPA) and p42/p44 MAP kinase. In these cells, OPNa and OPNb increased the levels of uPA, whereas OPNc did not promote this effect. Yet, OPNa and OPNb, but not OPNc, were able to increase the phosphorylation of ERK1/2 in hepatocarcinoma cellular cell lines. Together, these data suggest that OPNa and OPNb are involved in the activation of integrin signalling, but that OPNc cannot activate this pathway [37]. Besides, in head and neck cancer cells, the RGD region of OPNa and OPNb variants inhibited apoptosis by inducing NF-kappaB activation and FAK phosphorylation [58].

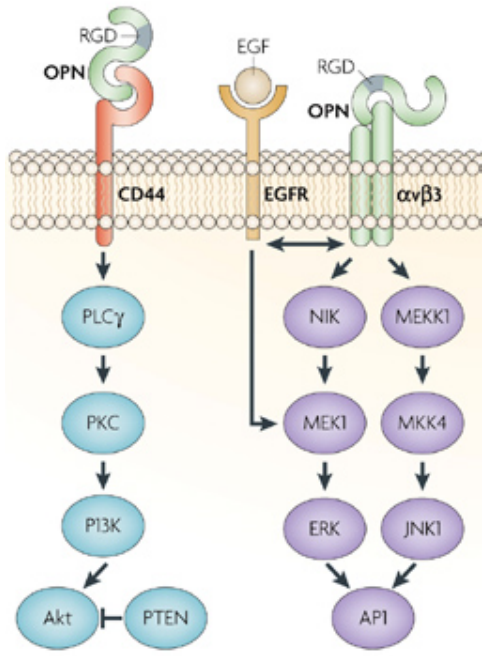

Figure 3: Activated OPN signalling pathways by binding to CD44 and integrin receptors. OPN can initiate Arg–Gly–Asp (RGD)-dependent and RGD-independent interactions with several integrins (such as αvβ3 and α9β1, respectively). OPN can also interact with the CD44 family of receptors. Some of these complexes are able to mediate the following functions: cell survival through phospholipase C-γ (PLCγ)–protein kinase C (PKC)–phosphatidylinositol 3-kinase (PI3K)–Akt pathway activation that leads to anti-apoptotic signals in tumour cells. OPN-induced Akt phosphorylation can be blocked by the tumour suppressor PTEN (phosphatase and tensin homologue). However, *PTEN* is frequently mutated and thus rendered inactive in cancer cells such as melanoma and glioma. Moreover, motility through the activation of the canonical αvβ3 integrin pathway where

both nuclear factor-inducing kinase (NIK)–ERK (extracellular signal-related kinase) and MEKK1 (also known as mitogen-activated protein kinase kinase kinase 1) (MAP3K1)–JNK1 (also known as MAPK8) signalling promote cell migration by activating AP1-dependent gene expression. Upon binding to αvβ3, OPN also stimulates epidermal growth factor receptor (EGFR) transactivation, ERK phosphorylation and AP1 activation. Adapted from: Bellahcène et al., 2008 (Nature Reviews Cancer).

1.1.8 OPN in normal tissues

In human, OPN is one of the most abundant non-collagenous, non-specific protein in bone. It is expressed in a variety of tissues such as bone, bone marrow derived gland cells, cartilage, dentine, cementum, kidney, brain, vascular tissues, epithelial cells of the gastrointestinal, urinary and reproductive tracts, the gall bladder, pancreas, lung bronchi, lactating breast, salivary glands, sweat ducts as well as in activated macrophages and lymphocytes. Furthermore, OPN is found in biological fluids, such as milk, urine, blood and seminal fluid [59]. Because OPN was mainly found to be expressed in the luminal surfaces of these tissues, some authors suggested that OPN might have a protective role in interactions between epithelial surfaces and the external environment [60]. For instance, it was described that OPN is likely to have a physiologic role in milk [61].

Cell growth, differentiation, and a high degree of tissue remodelling occurs during the different stages of the mammary gland. During pregnancy and lactation, these processes ensure the establishment of the spatial relations between stromal and epithelial cells, and the organization of the latter into a branched tree of ducts and terminal alveoli. The highly metabolic active epithelial cells lining the ducts rest on a basal membrane of collagen and other ECM proteins, and signals from the ECM to the cells are important for cell differentiation and milk secretion [62]. Studies on the expression and regulation of OPN in the mammary glands has been described by several investigators. Experiments showed that mammary epithelial cells that had the levels of OPN down-regulated by antisense mRNA, showed an increase in matrix metalloproteinase MMP-2. Possibly, MMP-2 and OPN compete for binding to integrins in the cells, and OPN binding can induce normal cell differentiation, whereas MMP-2 binding induces tissue degradation [60].

1.1.9 OPN in cancer

Cancer is a multifactorial pathological disease, emerging from deregulation of one or more cellular phenomena, resulting from accumulation of aberrant genetic and/or epigenetic alterations [63]. The cancer environment is comprised of tumour cells as well as a wide network of stromal and vascular cells that participates in the cellular and molecular events necessary for invasion and metastasis. Hence, cancer cells are regulated not only by autonomous signalling pathways, but also by contextual molecular interactions received from tumour-associated stroma [64].

OPN is a multifunctional matricellular protein and its capacity to bind to receptor complexes and activate different signalling pathways leading to a variety of effects such as cell survival, proliferation, migration and invasion, have been clinically and functionally associated with many types of cancers [65]. It has been well stablished that OPN is important for tumour initiation and invasion in liver, gastric, colorectal, lung, ovary and prostate cancer [66, 67].

Moreover, OPN may induce changes in the expression of many genes, resulting in proteins that can contribute to altered cell behaviour, including migration and invasion, an effect promoted by interactions of OPN with cell surface receptors. These effects of OPN may vary between cell types, depending for example on which integrins are expressed and which signal pathways can be activated. There is convincing evidence that soluble OPN, in several situations, help cells to survive a situation which would be lethal [60]. Notably, this survival signalling is mediated by receptors that are generally considered to be receptors for ECM components. It has been suggested that OPN delivers an anti-apoptotic "ECM-like" signal via multiple ligand-receptor interactions to both adherent and non-adherent cells [68]. Multiple and complex mechanisms are involved in OPN roles in cancer, not only interactions with cell surface receptors, but also proteases and growth factor/receptor pathways [60].

A huge amount of studies has indicated that OPN is highly expressed in several malignancies, including cancer. Abundant secreted OPN act as a marker for osteosarcoma, glioblastoma, prostate and breast cancer, squamous cell carcinoma and melanoma [69]. Additionally, OPN might also contribute to cancer progression by regulating the tumour-surveillance mechanism and inhibiting apoptosis of neoplastic

cells [70]. In cancer, the mechanisms through which OPN exerts its effect are incompletely understood. Recent studies pointed to a link between OPN and the epithelial-mesenchymal transition (EMT), appearing to be a significant molecular explanation for how OPN influences cancer development [71].

1.1.10 OPN and calcification

OPN is known as a "molecule for all seasons" because of its multifunctional roles. Apart from its functions related to cell survival, migration, proliferation, invasion, metabolic regulation, inflammation, etc; many studies have reported the importance of OPN in bone microenvironment [72]. Accordingly, OPN is involved in bone remodelling and mineralization. In healthy bone tissues, OPN is expressed by both osteoclasts and osteoblasts, which are responsible for bone remodelling. During physiological bone mineralization, osteoclast-derived OPN inhibits the formation of hydroxyapatite [73, 74]. Additionally, in teeth, OPN in the cement layer may be utilized for osteoblast cell adhesion, or to guide early calcification events at this tissue junction. The expression of iOPN, together with CD44, in proliferating osteogenic cells seems to be correlated with cell motility. Yet, OPN expressed during cement layer formation possibly regulate hydroxyapatite crystal growth, which would be consistent with the demonstrated ability of phosphorylated OPN as a potent regulator of hydroxyapatite crystal growth [73-75].

Moreover, in the urinary tract, OPN was demonstrated to be related with defence against renal stones formation, mostly composed by calcium oxalate. OPN is then synthesized within the kidney and secreted into the urine by epithelial cells [76, 77]. OPN can inhibit the nucleation, growth and aggregation of calcium oxalate crystals *in vitro* and directly inhibits the binding of calcium oxalate crystals to cultured renal epithelial cells [78]. Currently, the exact molecular mechanisms by which OPN exerts its many inhibitory effects in unclear, although it is known that phosphorylation of the OPN molecule is critically important [19, 20].

The functional diversity of OPN in bone formation and remodelling appears as fundamental roles of this protein in host defences and tissue repair. The bone remodelling sequences have many features of repair processes involving inflammatory

responses. Thus, a primarily role seems to be to facilitate organism recovery after injury or infection, which generally causes an increase in its expression. OPN stimulates cellular signalling pathways *via* various receptors found on most cell types, including the cells of mineralized tissues. It can regulate cell proliferation and phagocytic activity, and can both promote and participate on cell migration. Moreover, modulation of immune and inflammatory responses by OPN is reflected on the formation and activity of macrophages and also osteoclasts. As an ECM component, it can regulate mineral crystal formation and growth, and can form supramolecular structures by covalent interactions with other matrix macromolecules [14].

Although many studies have reported the altered expression of OPN in bone tumours, the mechanisms by which OPN overexpression are related with calcification in different pathologies and in tumour microenvironment, is still poorly understood. For instance, the up-regulation of OPN is also found in regions of dystrophic calcification such as that associated with degenerative and atheromatous vascular disease [79, 80].

The mechanism underlying the formation of calcifications in PTC is incompletely known. Underwood [81] believe that the psammoma bodies (PB) represent a process of dystrophic calcification, in which the deposition occurs locally in nonviable or dying tissues with normal serum levels of calcium, in the absence of derangements of calcium metabolism. Cameron and Mc Cluggage [82] however, hypothesized that PB formation is the result of a factor secreted locally by the tumour cells, rather than the widely held theory that their formation is secondary to necrosis, with subsequent dystrophic calcification within a papillary neoplasm. However, Kozlovskii *et al*. [83] in serous ovary adenocarcinoma (where PB are also frequent) suggested that PB were formed intracellularly, resulting in cell death and release of small PB. Tsuchida *et al*. [84] in meningioma described formation of precursors of PB extracellularly, due to collagen production by meningothelial cells. Despite the lack of a consensus regarding the mechanisms by which PB are formed, literature data indicate that OPN may be involved in these processes. Tunio *et al*. [85] observed PTC cells overexpressing OPN mRNA around PB. In addition, the localization of OPN protein expression was also consistent with that of PB. The authors further reported that the OPN transcript-expressing cells were identified as $CD68^{+}$ macrophages. In a more recent study, OPN expression presented a significantly association with the presence of PB in PTC samples, together

with the expression of the bone sialoprotein (BSP). The authors advanced that the strong correlation between BSP, OPN and PTC suggest a role for BSP and OPN on calcification and PTC tumour progression [86].

1.1.11 OPN in bone metastases

Concerning bone metastases, OPN expression has been detected in both osteoclasts and metastatic tumour cells [87]. Kang *et al.* [88] showed that breast cancer cells spreading to bone had increased OPN expression and identified OPN in a bone metastasis gene signature. Consistent with this finding, Nemoto *et al*. [89] reported that melanoma cells with down-regulated OPN have lower incidence of bone metastases. Moreover, OPN expression may differ between bone metastases with a more osteolytic *versus* osteoblastic pattern of involvement [90]. For example, tumour cells in bone metastases from breast cancer (mixed osteolytic and osteoblastic/sclerotic pattern) have strong OPN expression, compared to lower OPN expression in bone metastases from prostate carcinomas (more osteoblastic/sclerotic pattern) [90].

A number of studies have demonstrated that OPN may be involved in early tumour cell colonization into the bone [91]. Yoneda *et al*. [92] showed that the interaction between tumour cells and bone marrow endothelial cells may be an early step in bone metastasis formation. Breast tumour cells without OPN have decreased ability to bind bone marrow endothelial cells [91, 93]. OPN expression by prostatic tumour cells is also induced because of physical contact with bone marrow stromal cells [94]. Once contact among cancer cells, bone marrow stromal cells and bone marrow endothelial cells is important for both survival and proliferation, OPN expression may be important for cancer metastatic growth in bone microenvironment. OPN also helps to regulate osteoclast activation and adhesion to the bone surface, activating the mechanism of bone resorption, which aids on bone metastasis formation [87, 95, 96]. Thus, OPN expressed in tumour cells that have spread to bone, as well as in a number of cell types in the bone itself, may play a functional role on helping cancer cells survival and growth within the bone microenvironment.

1.1.12 OPN in thyroid cancer

OPN was first described to be overexpressed in human TC by Brown *et al*. in 1994 [97], in a study that evaluated OPN expression and distribution in human carcinomas when compared to corresponding normal tissues. In this report, by using *in situ* hybridization, OPN transcript was demonstrated to be expressed in macrophages closely associated with tumour cells, which contained no detectable OPN mRNA expression [59]. In 1998 the expression of OPN was associated with the presence of PB in human papillary thyroid cancer (PTC), and these cells were identified as $CD68^{+}$ macrophages. The authors concluded that OPN produced by macrophages may play a significant role in the development of PB in PTCs [85]. In 2003, OPN was demonstrated to be up-regulated by thyrotropin (TSH) hormone and iodine in cultured human thyroid follicles, using a cDNA microarray experiment [98]. Later, in a more complex study, Castellone *et al*. [99] showed that OPN is a transcriptional target of RET/PTC- in PCCl3 thyroid follicular rat cell line. They also demonstrated that OPN overexpression increased proliferation and invasion of RET/PTC-transformed PCCL3 cell line. After that, Guarino *et al*. [100] reported overexpression of OPN in a much large series of human PTCs, and such overexpression was correlated with the presence of lymph node metastases and tumour size. They also showed that the treatment of human PTC cell line with recombinant OPN also enhanced cell invasiveness. Briese *et al*. [101] analysed OPN expression patterns in thyroid carcinomas and they observed OPN overexpression in PTC and in MTC tissue samples. Further work associated OPN up-regulation to the occurrence of microcalcification and lymph node metastasis, suggesting a possible role of OPN in PTC calcifications [86, 102]. Recently, Kang KH [103] showed an association between OPN up-regulation, $BRAF^{V600E}$ mutation and lymph node metastasis in PTC samples.

1.2 Tumour microenvironment

1.2.1 Extracellular Matrix (EMC) and tumour microenvironment (TME)

The ECM is the non-cellular component, a collection of extracellular molecules secreted by cells in all organs and tissues, providing essential physical scaffolding for the cellular constituents and supplying crucial biochemical and biomechanical structure to support tissue morphogenesis, differentiation and homeostasis [104]. The ECM macromolecules are assembled into three-dimensional structures and regulate cell growth, motility and survival by binding specific cell surface receptors such as integrins, syndecans and discoidin [105, 106]. Additionally, the ECM is also important to maintain growth factors and cytokines availability, and also regulates hydration and pH of the local microenvironment [107]. Yet, it is known that interactions between specific collagens and glycoproteins with cell receptors is important to control cellular cytoskeleton, differentiation and gene expression. Moreover, physical forces, generated by cellular interactions with the ECM can in turn influence proliferation and migration in normal tissues, but are currently in the spotlight with respect to tumour stroma and progression [108]. In 2011, Hanahan and Weinberg [63] revisited the hallmarks of cancer adding two emerging features: dysregulation of cellular metabolism and evasion of immune destruction. Importantly, the ECM regulates many of the same cellular responses that characterize these cancer hallmarks.

ECM has been widely described as an essential component of a typical tumour microenvironment (TME), which also comprise many other essential elements in the stroma including fibroblast, myofibroblasts, neuroendocrine cells, adipose cells, immune and inflammatory cells, the blood and lymphatic vascular networks [109]. Microenvironment and tumour cells mutually influence each other during tumour initiation and progression [110]. Therefore, TME is a crucial factor for the faith of any cancer. Recent reports have indicated that the TME can dictate aberrant tissue function and play a critical role in the subsequent development of more advanced and refractory malignancies [111]. Moreover, fibres of ECM proteins such as collagens seems to have an important role in tumour progression, building migration tracks for the tumour cells. At the same time, the ECM can function as a barrier blocking, e.g., the penetration of

immune cells into the tumour, or it can create a high interstitial fluid pressure preventing the perfusion of drugs, which facilitates chemoresistance [107].

The degradation and remodelling of the ECM are some of the essential steps involved in cell migration and tumour progression, being such steps critically dependent on the proteolytic activity generated by metalloproteinases (MMPs) and the plasminogen-activator–plasmin system [49]. The MMP enzymes are ECM-degrading molecules that play a crucial role in embryogenesis, tissue remodelling, inflammation and angiogenesis [112]. MMP2 and MMP9 (also called gelatinase A and B) are important contributors to the process of invasion, tumour growth and metastasis [113]. Both MMP2 and MMP9 efficiently degrade native type IV and V collagens, fibronectin, ectactin and elastin. Several studies have shown a correlation between MMP2 activation and metastatic potential and have suggested that MMP2 might act as a useful marker for the prognosis or diagnosis of various cancers [114].

1.2.2 Osteopontin in ECM

It has long been known that OPN can be produced by a diversity of cells that are present in the TME, including tumour, macrophages and stromal cells; however, its several contributions to tumour growth, progression and the TME are still incompletely understood. Studies of genetically manipulated animal models and the use of transformed cell lines have revealed important clues about the role of OPN in the TME [91].

As aforementioned, OPN can be expressed both by tumour cells and by many other cells that compose the TME. For instance, Tuck *et al*. [115] reported that OPN transcript and protein were detected in tumour cells and in tumour infiltrating inflammatory cells in a cohort of lymph node negative breast cancer patients. Similarly, Wang-Rodriguez *et al*. [116], observed OPN cytoplasmic staining in breast cancer cells and OPN expression in tumour-infiltrating inflammatory cells, whereas stromal fibroblasts and endothelial cells were negative for OPN expression. Another study demonstrated that in pulmonary artery sarcomas, tumour cells as well as macrophages and ECM were positive for OPN expression, being tumour cell staining most frequent at cell surface and at sites of invasion [117]. In cutaneous squamous cell carcinoma Chang *et al*. [118] found that in most cases, OPN was expressed in cancer cells and in ECM

adjacent to the tumour, but not in other stromal regions. Conversely, in some tumour types, OPN expression was found primarily in macrophages and stromal cells, and less frequently in the tumour cells themselves, as in oesophageal adenocarcinoma [119].

Thus, is not clear whether tumour-derived OPN is incorporated into the ECM. In some tissues, such as bone, OPN is an established component of the ECM and is important in cell attachment [120]. Although OPN has been found in the ECM of several cancer types by immunohistochemistry [117, 121, 122], it has been discussed that at least in some instances, tumour-derived OPN may be more soluble and not incorporated into the ECM [123]. Hence, it is undefined whether it is tumour- or stromal-derived OPN (or both) that can be incorporated into the ECM and affect tumour growth and progression. Although some evidences point that differences in OPN variants and PTMs may affect different OPN functions [124, 125], there is very scant data in literature on how tumour-derived OPN may differ from stroma-derived OPN, either structurally or functionally.

So far, it is also observed that OPN cellular staining patterns may change according to tumour stage. For instance, in colon carcinomas, well-differentiated tumours show OPN immunostaining at the apical cell surface, while poorly differentiated tumours OPN is expressed on the basal cell surface, at the stromal interface [97].

Finally, Figure 4 shows how OPN can contribute to a diversity of steps in tumour progression and metastases. OPN is likely to regulate key-molecules in order to facilitate matrix degradation, cell migration and invasion, then culminating on the establishment of tumour metastasis.

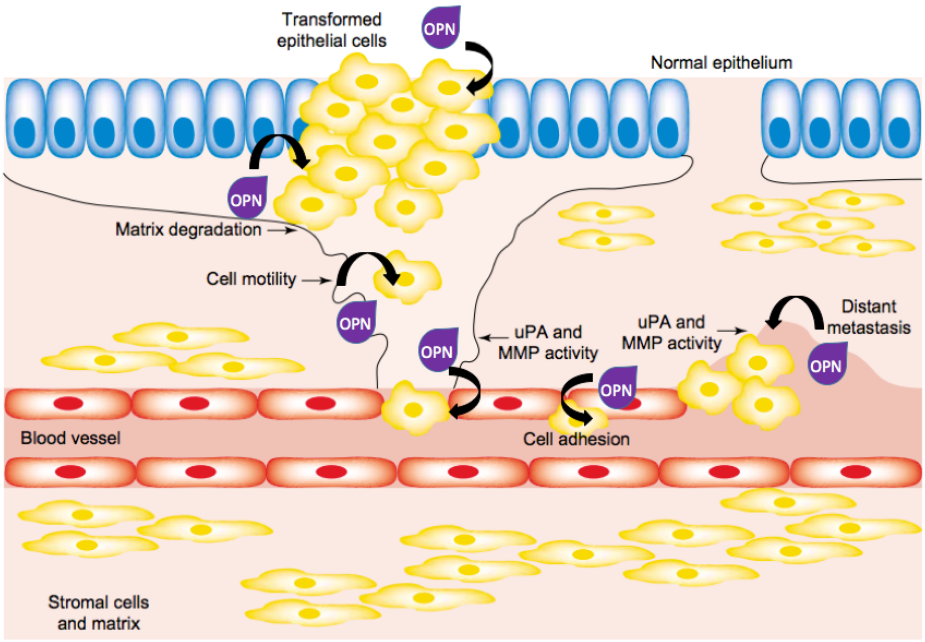

Figure 4: Model depicting the role of OPN in the regulation and activation MMPs during tumour progression and metastasis. Cancer metastasis is a highly complicated process that involves cell motility, invasion, angiogenesis, intravasation of tumour cells into the blood stream and subsequent extravasation, followed by colonization of tumour cells at secondary sites. The invasion involves translocation of neoplastic cells across tissue boundaries and through the extracellular matrix barrier. This process requires cellular adhesion and local proteolysis, leading to migration of neoplastic cells. Adapted from: Rangaswami *et al*., 2006. Trends in cell biology.

1.3 Thyroid Cancer

Thyroid cancer (TC) is the most common endocrine cancer, and its incidence has continuously increased in the last three decades all over the world [126].

Most of the thyroid cancers derive from follicular cells and a minority from C-cells. Based on recent data, TC is the fifth most common cancer in women [127]. In Portugal, a significant, rapid and continued increase in incidence has been observed for both sexes [128]. In the USA, approximately 1.0% – 1.5% of all new cancers diagnosed each year corresponds to TC [129]. Only in few countries (Norway and Sweden) TC incidence has decreased [130].

This significant increase in TC incidence has been largely attributed to the increasing use of ultrasonography and other imaging techniques, although environmental factors may also be important. It was found many years ago that small papillary carcinomas were a common finding at autopsy, reaching a frequency of 36% [131], suggesting that there is a large reservoir of small papillary carcinomas that do not present clinically during life, but can be uncovered by ultrasonography or other screening techniques [126]. A epidemiological study in Portugal, refers that the significant increased incidence is predominantly observed among women from the North of the country [128].

On the other hand, mortality rates from TC has minimally changed over the past five decades. TC present a broad range of clinical behaviours— in most cases indolent tumours with low mortality, and rarely, very aggressive malignancies, as anaplastic TC. The challenge faced by physicians who treat TCs is to balance the therapeutic approach so that patients with lower risk disease or benign thyroid nodules are not over treated. At the same time, they need to recognise those patients with more advanced or high-risk disease, who need a more aggressive treatment approach. Therefore, undertaking a proper diagnostic work- up before treatment is started, is crucial to appropriately tailor therapy.

Tumours originated by follicular cells can go through follicular adenomas (FTA), a benign encapsulated follicular lesion, to well-differentiated carcinomas (WDTC), such as papillary carcinoma (PTC) and follicular carcinoma (FTC) and finally, they can develop into poorly differentiated thyroid carcinoma (PDTC) and in anaplastic carcinoma (ATC - also called as undifferentiated cancer) as shown on Figure 5. The concept of stepwise

progression from a pre-existing WDTC to PDTC and ATC is supported by clinical, epidemiologic, pathologic and molecular evidence (studies of comparative genomic hybridization, loss of heterozygosity, genetic and epigenetic alterations). Yet, the PDTC and ATC can be originated independently from pre-existing papillary or follicular carcinomas and may apparently arise *de novo* [132-135].

Additionally, parafollicular cells can develop into the medullary thyroid carcinoma (MTC).

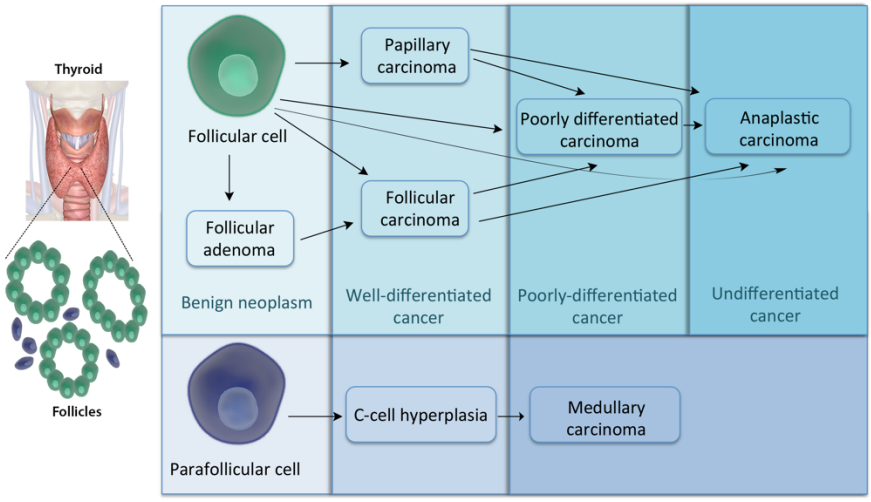

Figure 5: Schematic model of thyroid cancer progression. This model is based on histological and clinical features as well as on the degree of tumour differentiation. The WDTC cell may give rise to both benign and malignant tumours. Additionally, the differentiated thyroid follicles can also give rise to well-differentiated papillary or follicular carcinomas, PDTC, and anaplastic carcinoma. The parafollicular cells may give rise to C-cell hyperplasia, and then to MTC.

1.3.1 Well-differentiated thyroid cancer

1.3.1.1 Clinical Presentation

Among thyroid tumours, the well-differentiated carcinomas are the ones that presents the best overall prognosis. They are the most common tumours, accounting for more than 95% of cases and include three main entities: papillary thyroid carcinoma (PTC), follicular thyroid carcinoma (FTC) and Hürthle cell carcinoma (HCC).

PTC is the most common subtype and carries the best overall prognosis. Metastases most commonly involve cervical lymph nodes and, less commonly, the lungs. Microscopically, PTC are characterized by the presence of papillary areas, showing evidence of follicular cell differentiation, being characterized by distinctive nuclear features [136], such as large and clear nuclei, containing cytoplasmatic inclusions and grooves [137]. PTC is also associated with the presence of psammoma bodies (PB), which can appear as microcalcifications on ultrasonography in up to 50% of patients. Due to their well-differentiated nature, PTC cells often retain their ability to concentrate iodine and occasionally produce thyroid hormones [138-140]. They also produce and secrete thyroglobulin (Tg) and can express thyrotropin receptors on their surfaces [141]. PTCs that are composed totally or in part by papillae besides the aforementioned nuclear features, are classified as classic variant of PTC (cPTC), comprising the most frequent histotype.

The second most common histotype of PTC is the follicular variant (fvPTC), with an increasing frequency, comprising about 20-30 % of all PTCs. It is composed exclusively or almost exclusively of follicles lined by cells showing nuclear features of papillary carcinoma. Well-formed papillae should not be observed in fvPTCs [136, 142-144]. This variant commonly presents as infiltrative (or nonencapsulated) and as a well circumscribed or noninvasive encapsulated follicular variant of papillary thyroid carcinoma (EFVPTC), and as a result of encapsulation and follicular architecture, it may be mistaken for follicular adenoma or follicular carcinoma. Although fvPTC is distinguished from follicular adenoma and carcinoma by its characteristic “papillary” nucleus, nuclear features are often subtler than that of cPTC, making this variant one of the most controversial entities of surgical pathology, largely due to the lack of consensus on when to (or not to) diagnose the nuclear features of papillary carcinoma [143-146]. The EFVPTC has an indolent behaviour and is genetically distinct from infiltrative tumours. Yet, most patients with EFVPTC continue to be treated similarly to those with conventional PTC. Recently, Nikiforov et al. [147] proposed that noninvasive EFVPTC having a very low risk of adverse outcome should be termed as “noninvasive follicular thyroid neoplasms with papillary-like nuclear features” (NIFTP). This reclassification intends to affect a large population of patients worldwide to achieve a significant

reduction in psychological and clinical consequences associated with the diagnosis of cancer.

The EFVPTC rarely metastasises to lymph nodes, present only in about 5% of cases. The infiltrative/diffuse fvPTC tumours harbour more often lymph node metastasis, which are reported in around 65% of cases. The prognosis of these tumours is similar to the cPTC [148]. There are several other histological subtypes of PTC described, besides the cPTC and the fvPTC, but with low incidence. They always present the characteristic nuclei, therefore the variants of PTC differ in their morphologic organization and background [136].

The FTC is an unusual tumour comprising approximately 5 – 10% of thyroid malignancies in nonendemic goiter areas of the world [149]. The identification of capsular or vascular invasion differentiates FTC from follicular adenoma. Macroscopically, FTC tends to comprise solitary tumours of varying sizes with thick fibrous capsules that have a tan cut surface. FTC is considered a more aggressive WDTC when compared to PTC [150]. Several prognostic features that decrease the overall survival of FTC include age, tumour size, distant metastasis, extrathyroid extension and histologic type. In high risk groups (age > 45 years, stage > T1, distant metastasis, high histologic grade), the 10-year survival rate can be as low as 56% [151]. The two main subgroups of FTC are the minimally invasive and the widely invasive. Minimally invasive FTCs have been shown in several studies to have a more favourable prognosis [151-154]. FTC has a higher rate of haematogenous spread than PTC. It is important to the surgeon to recognize it at the time of diagnosis of FTC, once 10% to 15% of cases present distant metastasis [155-157].

HCC can be considered a subtype of FTC, accounting for 3-4% of TC and is pathologically characterized by the presence of cells rich in mitochondria (Hürthle or oncocytic cells). The Hürthle cell's rich mitochondrial content makes HCCs appear brown on their cut surface.

1.3.1.2 Genetic Alterations

The *BRAF*^{V600E} mutation is the most common genetic mutation detected in patients with TC and occur in approximately 50-70% of patients with PTC [158]. Genomic

rearrangements also occur often in TC leading to expression of novel fusion oncogenes that are present in many TC. PAX8–peroxisome proliferator- activated receptor γ (PPARγ) translocation occurs in more than 30% of FTC cases [159], and the RET-PTC translocations targeting the *RET* oncogene occur in about 20% of PTC cases [160]. Additionally, mutations in the promoter of telomerase (p*TERT)* have been identified in all types of TC, in particular in more aggressive subsets of TC [161].

1.3.1.3 Assessment and Treatment

The assessment of a thyroid nodule is made primarily by ultrasonography, and depending on such sonographic appearances, patient undergo (or not) to fine-needle aspiration (FNA). 2015 ATA guidelines recommend biopsy only for nodules larger than 1 cm. For the classification of the FNA, the Bethesda system for reporting thyroid cytopathology includes six diagnostic categories [162] that goes from benign to malignant nodules (which generally need surgery).

Concerning the treatment, PTC greater than 2 cm (T2) is best managed by total thyroidectomy. Thyroid lobectomy and isthmectomy may be adequate for unifocal PTC less than 1 cm in patients without negative prognostic factors. Central compartment and possible lateral neck dissections should be performed when nodal metastases are present in the respective nodal basins. Post-operatively, radioactive iodine (RAI) ablation with 131I followed by thyroid stimulating hormone (TSH) suppression is indicated in certain patients to improve loco-regional control and reduce recurrence [163].

FTC is diagnosed microscopically through the identification of capsular or vascular invasion. Finally, the microscopically diagnosis of HCC is made through identification of capsular or vascular invasion, similar to that of FTC, and the presence of Hürthle cells. Although HCC rarely presents lymph node metastases, it has the highest incidence of distant metastasis of all the WDTC. Approximately 34% of patients will develop distant metastasis at some time during their illness [164]. It also has a higher rate of refractoriness to radio-iodine therapy compared to PTC and FTC [156, 165].

1.3.2 Medullary thyroid carcinoma

1.3.2.1 C-cell pre-neoplastic lesion

C-Cell hyperplasia (CCH) was first described in the early 1970's [166-168] in asymptomatic relatives of patients with MTC. CCH is characterized by an abnormal increased number of C cells (parafollicular cells) within the thyroid gland.

Such hyperplasia can be further classified as physiological (reactive) or neoplastic [169]. The reactive CCH corresponds to proliferation of calcitonin-producing cells in response to different physiological and/or pathological endocrine stimuli (e.g., thyroid-stimulating hormone overstimulation, hypercalcemia, and paracrine factors) [170, 171]. Reactive CCH has no malignant potential and can be observed in association with many other thyroid diseases (including WDTC) [169, 172]. On the contrary, neoplastic CCH should be considered as a pre-neoplastic stage in the spectrum of C-cell disease, ultimately leading to the development of the MTC. In particular, the progression from CCH to MTC is a well-defined event in the hereditary setting, including isolated familial MTC and multiple endocrine neoplasia type 2 (MEN2) syndromes [171, 173, 174].

Neoplastic CCH is considered as a pre-neoplastic condition, commonly observed in patients with germ-line mutations in the *RET* oncogene (commonly in families with a history of hereditary MTC, i.e. familial MTC or multiple endocrine neoplasia types IIA and IIB) [174, 175]. In sporadic cases, the role of CCH in carcinogenesis is controversial. In such cases, CCH has been considered as a different state in C-cell pathology [176, 177].

Frequently, the diagnosis of CCH may fail, first because of the very low proportion of C-cells in thyroid, and secondly due to its particular form of distribution throughout the thyroid parenchyma (its density is higher in the upper two-thirds of the thyroid lobes, with the highest concentration found in the junction zone between the upper and middle thirds of each lobe [178, 179]); therefore, the partial examination of the thyroid gland may lead to non-representative material, causing diagnosis difficulties [178, 179].

Since C-cells are widely recognized for its ability to secrete calcitonin, prior to surgery, the CCH diagnosis should be suspected in the presence of hypercalcitoninemia.

Apart from MTC, CCH is the most common feature found in patients with hypercalcitoninemia, especially in the absence of suspect thyroid nodules (which could indicate the presence of MTC) [180, 181]. Overall, total thyroidectomy (which is currently the preferred surgical approach in the majority of thyroid diseases, including MTC), is an adequate treatment and achieves cure, even in patients with neoplastic CCH. However, for patients with pure CCH, cervical lymph node dissection is not performed [182].

1.3.2.2 Clinical Presentation

MTC is a rare thyroid malignancy (accounting for 1–2% of all TCs) [183]. Derived from C-cells, MTC is associated with a higher incidence of distant metastasis and poorer prognosis compared with the more frequent well-differentiated papillary and follicular thyroid carcinomas [184, 185]. This malignancy is often found as a single nodule in the thyroid in patients between forty and sixty years old [186]. Occasionally, neck lymphadenopathy is the first manifestation, because the disease frequently metastasises to cervical lymph nodes, being that 70% of the patients presenting palpable MTC have evidence of cervical metastases at surgery [187].

Due to the typical distribution of C-cells throughout the thyroid parenchyma, mentioned in the previous section, the tumour is typically located at the junction of the upper third and the lower two-thirds of the thyroid lobes. 25% of MTCs occur as hereditary forms, the majority of which occur as part of the multiple endocrine neoplasia (MEN) 2 syndromes [188].

1.3.2.3 Genetic Alterations

The most frequent mutations in MTC occur in the *RET* proto-oncogene, whereas a small proportion presents sporadic *RAS* mutations. *RET* mutations can be somatic or germline events exhibiting autosomal dominant inheritance [186, 189]. *RET* germline mutations can predispose patients to early development of MTC as a component of the MEN 2A and 2B syndromes. As such, patients who present MTC at young age are likely to have hereditary disease.

Somatic mutations of the *RET* proto-oncogene have been identified in 25-33% of sporadic MTC, and may be associated with a poor outcome compared with sporadic tumours without *RET* mutation [190, 191]. Besides mutations in different codons of *RET* gene, small deletions of the *RET* gene have also been identified in a few cases.

Because 1–7% of patients presenting apparently sporadic MTC are carriers of germline *RET* mutations [186], assessment for a heritable *RET* germline mutation should be recommended to all patients presenting MTC, regardless of their family history or age.

1.3.2.4 Assessment and Treatment

Surgery is the only curative treatment for MTC; however, few patients with clinically apparent nodal metastases at diagnosis achieve undetectable tumour markers. Imaging and diagnosis before surgery are crucial to indicate the appropriate surgical intervention, as with differentiated thyroid cancer. Ultrasonography and tumour markers (calcitonin and carcinoembryonic antigen) measurement are made in patients with preoperative diagnosis of MTC.

Such cases in which the tumour markers are undetectable and present normal imaging after surgery should continue to be followed up annually; those with persistent tumour markers need to be followed up more closely for progression. Calcitonin and carcinoembryonic antigen doubling times are useful measures, because they are predictive of outcomes and aggressive tumour behaviour [192]. Patients with calcitonin and carcinoembryonic antigen doubling times within 6 months have a shorter overall survival. Concerning chemotherapy, treatment with dacarbazine, 5-fluorouracil and doxorubicin (alone or in combination) has shown very limited efficacy, achieving only partial responses in the range of 10 e 20% and of short duration [193-195]. A variety of kinase inhibitors are currently under evaluation and preliminary results are promising. They include motesanib diphosphate, vandetanib, sorafenib and sunitinib. Preliminary results showed partial responses in 6-20% of patients, and stable disease in 47-87% of patients with tolerable and manageable toxicities [196-198].

Chapter 2 –Aims

With this thesis we intended to address the role of the total osteopontin (tOPN) and its variants (OPNa, OPNb and OPNc) in the two main histotypes thyroid carcinoma (TC). As mentioned above, OPN is a matricellular protein overexpressed in many types of cancers where it correlates with poor survival. OPN effects in tumour progression have been associated with its ability to induce ECM invasion and migration. Proteolysis and remodelling of the ECM represent early events modulating cancer cell invasion through the surrounding stroma, and OPN has been implicated in such processes. OPN has been shown to be overexpressed in TC being, similarly to what was reported in other tumour models, associated with guarded prognosis. Although some key molecular functions have been attributed to OPN in the invasion and progression of the thyroid tumours, little is known about the role of OPN in TC, in particular in what concerns the differential effect of OPN splice variants and the putative differences between the distinct TC histotypes.

Our aims were:

1) To evaluate the expression profile of tOPN, OPNa, OPNb and OPNc in TC tissues and to compare with their expression in samples from non-tumour at thyroid tissues and benign tumours;

2) To evaluate the relationship between tOPN expression profile and its variants with the clinicopathological data and genetic alterations of the two main types of TC;

3) To evaluate the functions of the OPN variants (OPNa, OPNb and OPNc) in TC cell lines and its relationship with their tumourigenic capacities, including the analysis of activity and profile of MMPs expression;

4) To evaluate the relationship between the expression of OPN and its variants with aspects of ECM remodelling, including the role of OPN in collagen deposition and the formation of dystrophic calcifications including psammoma bodies.

Chapter 3 – Paper I - Osteopontin-a splice variant is overexpressed in papillary thyroid carcinoma and modulates invasive behavior

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Research Paper

Osteopontin-a splice variant is overexpressed in papillary thyroid carcinoma and modulates invasive behavior

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ABSTRACT

Osteopontin (OPN) is a matricellular protein overexpressed in cancer cells and modulates tumorigenesis and metastasis, including in thyroid cancer (TC). The contribution of each OPN splice variant (OPN-SV), named OPNa, OPNb and OPNc, in TC is currently unknown. This study evaluates the expression of total OPN (tOPN) and OPN-SV in TC tissues and cell lines, their correlation with clinicopathological, molecular features and their functional roles. We showed that tOPN and OPNa are overexpressed in classic papillary thyroid carcinoma (cPTC) in relation to adjacent thyroid, adenoma and follicular variant of papillary thyroid carcinoma (fvPTC) tissues. In cPTC, OPNa overexpression is associated with larger tumor size, vascular invasion, extrathyroid extension and *BRAF*^{V600E} mutation. We found that TC cell lines overexpressing OPNa exhibited increased proliferation, migration, motility and *in vivo* invasion. Conditioned medium secreted from cells overexpressing OPNa induce MMP2 and MMP9 metalloproteinases activity. In summary, we described the expression pattern of OPN-SV in cPTC samples and the key role of OPNa expression on activating TC tumor progression features. Our findings highlight OPNa variant as TC biomarker, besides being a putative target for cPTC therapeutic approaches.

INTRODUCTION

Thyroid cancer (TC) is the most common endocrine malignancy, being the fifth most frequent cancer in women [1]. TC incidence rates have been increasing in the last three decades all over the world [2]. The reason for the raised incidence of TCs remains controversial. While some studies point to improved diagnostic approaches [3, 4], others indicate that it may be correlated to environmental and lifestyle changes [5-8].

The majority of TCs are derived from follicular cells, being differentiated thyroid cancer (DTC), comprising papillary thyroid cancer (PTC) and follicular thyroid cancer (FTC), the most common subtypes and accounting for 90–95% of all cases [9]. Within PTCs, which encompass more than 80% of TCs, classical type

of PTC (cPTC) corresponds to around 50% of the cases, whereas follicular variant PTC (fvPTC) corresponds to about 40% of all PTCs [10].

Despite the overall good prognosis of DTCs, a subset of DTC patients follows a more aggressive disease course, developing recurrent or metastatic disease [11]. The early identification of this cases is one of the most challenging tasks in thyroid oncology.

Osteopontin (OPN) is a secreted extracellular matrix (ECM) protein encoded by the highly conserved *SPP1* gene [12]. Previous studies have found that total OPN (tOPN) is overexpressed in TCs [13-17], similarly as reported in other tumor models, being correlated with poor survival [17]. tOPN effects in tumor progression has been associated with its ability to induce ECM invasion and migration [18]. Proteolysis and remodeling of the ECM represent early events modulating cancer cell invasion through the surrounding stroma [19, 20], and OPN has been implicated in such processes [21, 22].

OPN primary transcript is subjected to alternative splicing, generating three OPN splicing variants (OPN-SV): the full-length OPNa and the shorter variants OPNb and OPNc (lacking exons 5 and 4, respectively) [23]. Recent studies have shown that OPN-SV are differentially expressed and may exhibit functional differences in normal tissue and their respective tumors [24]. For instance [25], in hepatocellular carcinoma (HCC), tumor tissues predominantly expressed OPNa and OPNb, while normal liver tissues mainly expressed OPNc. In this tumor model, OPNa and OPNb induced Hep3B cell migration, while OPNc had no significant effect. However, in SK-Hep1 cells OPNc suppressed the migratory activity, while OPNa induced no significant changes [25]. Our group previously demonstrated that OPNc, but not OPNa and OPNb, is specifically expressed in ovarian cancer samples. Furthermore, OPNc overexpression in OvCar-3 cells activates proliferation, migration, invasion and colony formation, as well as tumor formation in nude mice [26].

Based on these data, we then hypothesized that specific OPN-SV could be putative biomarkers in TCs. We here investigated the expression patterns and putative biological roles of tOPN and OPN-SV in DTC tumor progression.

RESULTS

tOPN protein is overexpressed in cPTC and is associated with vascular invasion and extrathyroid extension

In thyroid tumor tissues (Figure 1A-1H), tOPN staining was mainly localized in the cytoplasm of TC cells, although a few samples also showed focal membrane staining (Figure 1G and 1H). Total OPN protein expression was observed in 27 of 44 cPTCs (61.4%), in 6 of 16 fvPTCs (37.6%) and in 6 of 10 FTCs (60%). In the cPTC, staining intensity was faint in 31.8%, moderate in 20.5% and strong in 9.1% of the cases (Table 1). Staining score of the positive cases was moderate to high in the majority of the cases (Table 1). Representative sections of tOPN staining scores from 0 to 7 are also depicted in Figure 1. Tissues adjacent to thyroid tumor areas were virtually negative for tOPN staining (Figure 1I and 1J).

Of note, cPTC samples displaying vascular invasion exhibited higher tOPN staining scores than tumors without vascular invasion (Table 2). Tumors displaying extrathyroid extension had higher average tOPN staining score than tumors without this feature, although not attaining statistical significance (Table 2). cPTC samples containing hyaline stroma exhibited higher tOPN staining score than tumors without stroma ($p = 0.01$) (Table 2). In cPTC samples, no significant associations were observed between tOPN staining scores and patient' gender or age, tumor size, capsular invasion, lymph node metastasis, thyroiditis, *RET/PTC* translocation, $BRAF^{V600E}$, *RAS* and *TERT* mutations. Moreover, no significant associations were observed between tOPN staining score and any clinicopathological or molecular features in FTC (data not show). With regard to tOPN protein expression in well and poorly circumscribed fvPTC cases, there is a difference in the tOPN staining score media, although not attaining statistical significance (Supplementary Table S1).

OPNa is the predominant OPN-SV expressed in cPTC tissues and thyroid cell lines and is associated to invasiveness

Since we had observed a higher tOPN protein expression in cPTC and a significant association with invasive features, we then evaluated the mRNA expression patterns of each OPN-SV (OPNa, OPNb or OPNc) and of tOPN, in an attempt to evaluate their relative contributions to cell invasiveness. The comparison of tOPN and each OPN-SV expression levels among distinct thyroid tissue samples showed that tOPN is overexpressed in relation to each OPN-SV (Supplementary Figure S1). It is worth to mention, that tOPN expression corresponds to the sum of all OPN-SV. Among the three OPN-SV, OPNa has the highest expression levels in all thyroid tissues (Figure 2A and Supplementary Figure S1).

cPTC samples express higher levels of tOPN, OPNa and OPNb, when compared to adjacent thyroid, adenoma and FTC samples ($p < 0.05$) (Figure 2A; 2B and 2C). OPNc variant has the lowest expression levels and is only significantly overexpressed in cPTC samples in relation to adenoma and fvPTC samples (Figure 2D). These data show that OPNa splice variant is overexpressed in cPTCs when compared to other thyroid tissues. In an attempt to understand the expression pattern of OPN-SV in fvPTC cases, we separately analyzed well or poorly circumscribed fvPTC cases. We observed that poorly circumscribed fvPTC significantly overexpress tOPN, OPNb and OPNc variants, compared with well-circumscribed cases of fvPTC ($p = 0.02$,

p = 0.002 and p = 0.01; respectively). Also a higher expression of OPNa in poorly circumscribed fvPTC was noted, although not reaching statistical significance (p = 0.07) (Figure 2E).

The median transcript expression levels of tOPN were significantly higher in cPTC tumors larger than 2 cm (Table 3). tOPN and OPNa median expression levels were significantly

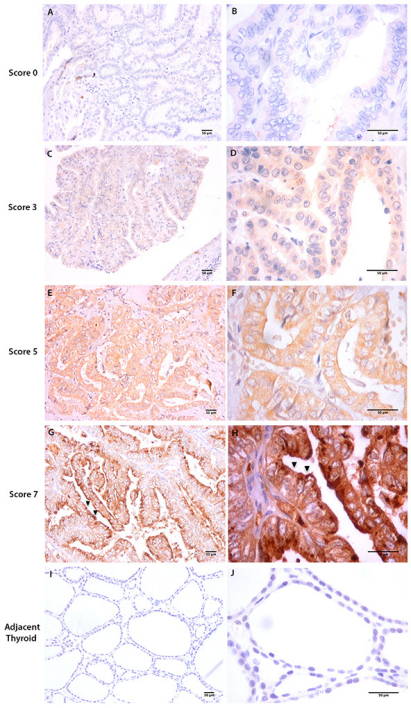

Figure 1: Total OPN (tOPN) IHC staining in cPTC samples. Representative sections of cPTC samples showing tOPN staining in thyroid tumor cells (20x and 60x magnification, respectively at the left and right images are shown **A-H.** Scale bar: 50 μm). A and B represent cPTC samples with staining score 0; C and D, staining score 3; E and F, staining score 5; G and H staining score 7. Black arrows points to membrane tOPN staining (G and H); **I** and **J**: represent adjacent thyroid tissues negative for tOPN staining.

Table 1: Staining intensity, proportion of positive stained cells and staining score of tOPN IHC in cPTC samples

Staining intensity	n	%	Proportion of positive stained cells %	n	%	OPN Staining Score*	%
Absent	17	38.6	**<5%**	22	50	0	38.6
						1	2.3
Faint	14	31.8	**5-25%**	6	13.6	2	15.9
						3	11.4
Moderate	9	20.5	**25-50%**	4	9.1	4	4.5
						5	13.6
Strong	4	9.1	**50-75%**	1	2.3	6	11.4
						7	2.3
			75-100%	11	25		
Total	44	100		44	100	44	100

* Staining intensity plus % of positive stained cells

Table 2: tOPN protein expression evaluated by IHC and correlation with clinicopathological associations in formalin-fixed paraffin-embedded (FFPE)

Tissue	Clinicopathological features (N)	tOPN tissue expression (Average ± SD)	p-value
cPTC	Stroma		
	Absent (n=17)	1.53 ± 2.18	**p=0.01**
	Hyaline (n=19)	3.37 ± 2.16	
	Vascular Invasion		
	Absent (n=16)	1.44 ± 2.15	**p=0.05**
	Present (n=23)	2.91 ± 2.41	
	Extrathyroid Extension		
	Absent (n=21)	0.67 ± 1.98	p=0.07
	Present (n=18)	3.00 ± 2.56	

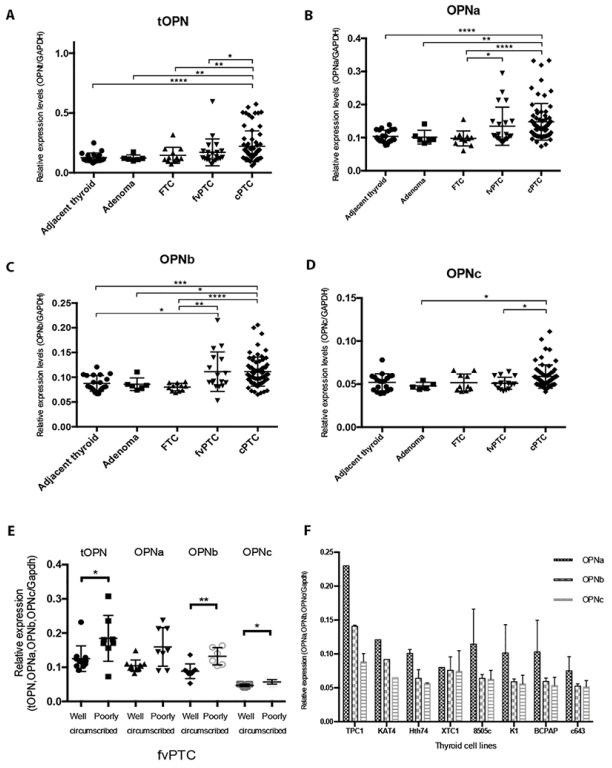

Figure 2: Transcript expression levels of tOPN, OPNa, OPNb and OPNc in thyroid tissue samples and in thyroid cell lines. A. tOPN, **B.** OPNa, **C.** OPNb and **D.** OPNc mRNA expression levels has been measured by real time PCR in the distinct thyroid tissue samples (symbols: adjacent thyroid -•; follicular adenomas -■; FTC -▲; fvPTC -▼; cPTC -◆); **E.** mRNA expression levels have been measured by real time PCR in well and poorly circumscribed fvPTC tissue samples (symbols: tOPN expression in well circumscribed fvPTC -•; tOPN expression in poorly circumscribed fvPTC -■; OPNa expression in well circumscribed fvPTC -▲; OPNa expression in poorly circumscribed fvPTC -▼; OPNb expression in well circumscribed fvPTC -◆; OPNb expression in poorly circumscribed fvPTC -○; OPNc expression in well circumscribed fvPTC - •; OPNc expression in poorly circumscribed fvPTC -Δ) **F.** OPN-SV transcript expression levels have been also evaluated in distinct thyroid tumor cell lines (TPC1, KAT4, Hth74, XTC1, 8505c, K1, BCPAP and c643). * $p < 0,05$; ** $p < 0,01$; **** $p < 0,0001$. Results are representative of at least two independent assays with triplicates.

Table 3: Correlation between tOPN and OPN-SV transcript expression levels with clinicopathological and molecular features in cPTC and fvPTC samples

Tissue	Variable	tOPN mRNA expression (Median)	p-value	OPNa mRNA expression (Median)	p-value	OPNb mRNA expression (Median)	p-value
cPTC	Tumor size (cm)						
	<2 (n=18)	0.14	**p=0.02**	0.12	p=0.27	0.10	p=0.89
	≥ 2 (n=38)	0.17		0.13		0.10	
	Extrathyroid Extension						
	Absent (n=20)	0.15	**p=0.04**	0.12	**p=0.02**	0.09	p=0.09
	Present (n=21)	0.18		0.13		0.11	
	Vascular Invasion						
	Absent (n=22)	0.15	**p=0.04**	0.12	**p=0.03**	0.10	p=0.45
	Present (n=28)	0.17		0.13		0.10	
	BRAF^{V600E} Mutation						
	Absent (n=26)	0.14	**p=0.03**	0.12	**p=0.01**	0.09	**p=0.01**
	Present (n=30)	0.18		0.13		0.11	
fvPTC	Age (yr)						
	<45 (n=13)	0.11	**p=0.03**	0.09	**p=0.04**	0.08	p=0.07
	≥45 (n=9)	0.17		0.14		0.11	

higher in cPTC samples with extrathyroid extension and vascular invasion than in those without such features (Table 3). cPTC harboring *BRAF*^{V600E} gene mutation exhibited higher tOPN, OPNa and OPNb transcript expression levels than those presenting wild type *BRAF gene* ($p < 0.03$) (Table 3). In fvPTC, older patients presented higher tOPN and OPNa expression levels ($p < 0.04$). No statistical significant differences have been observed between tOPN and OPN-SV transcript expression levels and other clinicopathological or molecular features in fvPTC.

Regarding OPN-SV expression patterns in TC cell lines, we found that OPNa variant is also overexpressed when compared to OPNb and OPNc variants in all the tested TC cell lines, except XTC1 (Figure 2F).

OPNa overexpression modulates proliferation and migration in c643 and 8505c cell lines

c643 and 8505c cells transfected with each OPN-SV express higher transcript levels of the corresponding ectopically expressed OPN-SV in relation to EV control cells (Figure 3A and 3B) and were used for further functional assays, as depicted below. The overexpression of OPN protein in c643 and 8505c cell lines transfected with tOPN and each OPN-SV was validated using immunocytochemistry (Figure 3C and 3D).

c643 and 8505c OPNa overexpressing cells displayed higher proliferation rates than OPNb, OPNc and EV control at 48 h ($p < 0.01$) (Figure 4A, 4B; $p < 0.05$).

In order to investigate the effect of OPN-SV overexpression on c643 and 8505c cell migration, these cells were subjected to *in vitro* wound closure assays (Fig. 4C, 4D, 4E and 4F). c643 clones overexpressing OPNa have higher migration rates, as depicted by wounding area, than OPNb, OPNc or EV clones. At 6h after cell scratch, c643-OPNa overexpressing cells completely closed the wound edges, at variance with OPNb, OPNc or EV clones. The same migration behavior was observed for 8505c-OPNa overexpressing cells, although the wound closure has only been achieved at 12h after cell scratch. In order to further validate these motility properties, we used time-lapse video microscopy, monitoring the distance travelled by the cells during 12h. As shown in Figure 4G, c643-OPNa overexpressing clones showed higher motility rates than the remaining OPN-SV clones and EV controls. Similar higher motility behavior pattern has been observed for 8505c-OPNa overexpressing cells (Figure 4H) (Representative videos are shown in Supplemental Material).

TC cells overexpressing OPNa induces MMP2 and MMP9 activity

We then investigated the impact of OPNa overexpression in invasion-related enzymes secreted at the extracellular conditioned medium (CM). CM secreted from c643 and 8505c cells overexpressing OPNa, OPNb, OPNc or EV control were tested for MMP2 and MMP9 metalloproteinase activity. We found

that the levels of matrix MMP2, mainly the active form, were increased in the CM secreted from c643-OPNa overexpressing cells, compared to CM secreted from the corresponding c643-OPNb and c643-OPNc clones (Figure 5A). No MMP9 expression was detected in c643 cells, regardless of the expressed OPN-SV. We observed an increase in the activity of MMP2 and MMP9 in the CM secreted from 8505c-OPNa overexpressing cells (Figure 5B). 8505c-OPNa overexpressing cells present higher levels of active MMP2 and MMP9 than 8505c-OPNb and 8505c-OPNc overexpressing cells.

Overexpression of OPNa increases the invasive potential of TC cells in the CAM assay

In vivo CAM assays were conducted to evaluate the effect of ectopic OPNa overexpression on the angiogenic, tumorigenic and invasive behavior of c643 cells. To achieve this, the c643 clones overexpressing OPNa and EV were injected in the chick embryo CAM.

Tumors formed by cells overexpressing OPNa exhibited an invasive pattern (Figure 6A, left). In contrast, xenograft tumors formed by c643-EV cells were compact, with encapsulated-like borders (Figure 6A, right), as evaluated by HE staining (Figure 6A, upper panel) and IHC for tOPN staining (Figure 6A, lower panel). We used a score system to semi-quantify the property of TC cells to spread inside the CAM. We observed that c643 cells overexpressing OPNa exhibited higher invasive capacity ($p = 0.003$) than c643-EV cells. This higher invasiveness was characterized by the presence of tumor cells oriented towards the invasion front and the presence of isolated cells and small clusters at distance from the tumor bulk (Figure 6B). Similar angiogenic ($p > 0.05$; Figure 6C) and tumorigenic responses ($p > 0.05$; Figure 6D) were observed in c643-OPNa when compared with c643-EV control.

DISCUSSION

We characterize for the first time the transcript and protein expression patterns of tOPN and OPN-SV in DTC

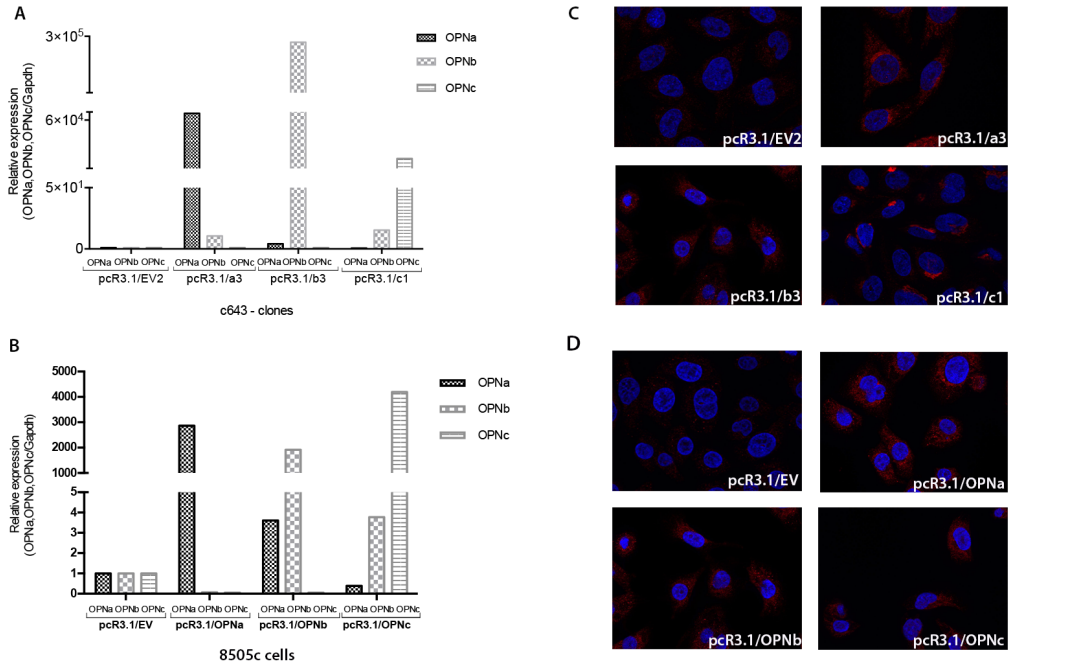

Figure 3: Stable overexpression of each OPN-SV in c643 and 8505c transfected cells. The expression levels of each OPN-SV were analyzed by qRT-PCR, when compared to control cells transfected with EV plasmid, which was used as the reference sample. The relative expression levels of each OPN-SV were calculated using the delta-delta CT method (pcR3.1/OPNa, pcR3.1/OPNb or pcR3.1/OPNc relative to pcR3.1/EV). Each OPN-SV is represented by a different bar graph, as indicated. **A.** OPN-SV mRNA overexpression in c643 cells; **B.** OPN-SV mRNA overexpression in 8505c cells. **C.** Immunocytochemistry analyses of OPN expression in control cells (c643 cells with EV: pcR3.1/EV) and in c643 cells overexpressing OPNa (pcR3.1/OPNa), OPNb (pcR3.1/OPNb) and OPNc (pcR3.1/OPNc) have been performed using the anti-tOPN antibody; **D.** Immunocytochemistry analyses of OPN expression in control cells (8505c cells with EV: pcR3.1/EV) and in 8505c cells overexpressing OPNa (pcR3.1/OPNa), OPNb (pcR3.1/OPNb) and OPNc (pcR3.1/OPNc). NOTE: Cell isolated clones used for these assays were named EV2, a3, b3 and c1 for each OPN-SV.

and TC cells. Then, we also showed their association to TC prognosis and progression features, especially in cPTC samples. Our data further demonstrated that OPNa transcript is the dominant overexpressed splice variant in DTC tissues and in distinct thyroid cell lines, notably in cPTC. Remarkably, high transcript expression levels of tOPN and OPNa, but not OPNb and OPNc, were associated with aggressive cPTC clinicopathological features (tumor size, vascular and extrathyroid invasion). Moreover, we demonstrated using *in vitro* and *in vivo* approaches that ectopically overexpressed OPNa promotes cell growth, migration and invasion in TC-derived cell lines.

We have shown that tOPN is predominantly expressed (both at transcript and protein level) in cPTC and in fvPTC, when compared to FTC and adjacent non-tumoral thyroid tissues. In fvPTC samples, we observed higher expression of tOPN, OPNa and OPNb variant in poorly circumscribed cases. This result is very interesting for several reasons: a) first, it is in accordance with the increased expression of OPN and the respective spliced forms in cases presenting invasive features; b) second, it corroborates our *in vitro* and *in vivo* results showing an increased invasiveness in cells overexpressing tOPN and OPN-SV; c) finally, it shows the different biological characteristics of the well and poorly

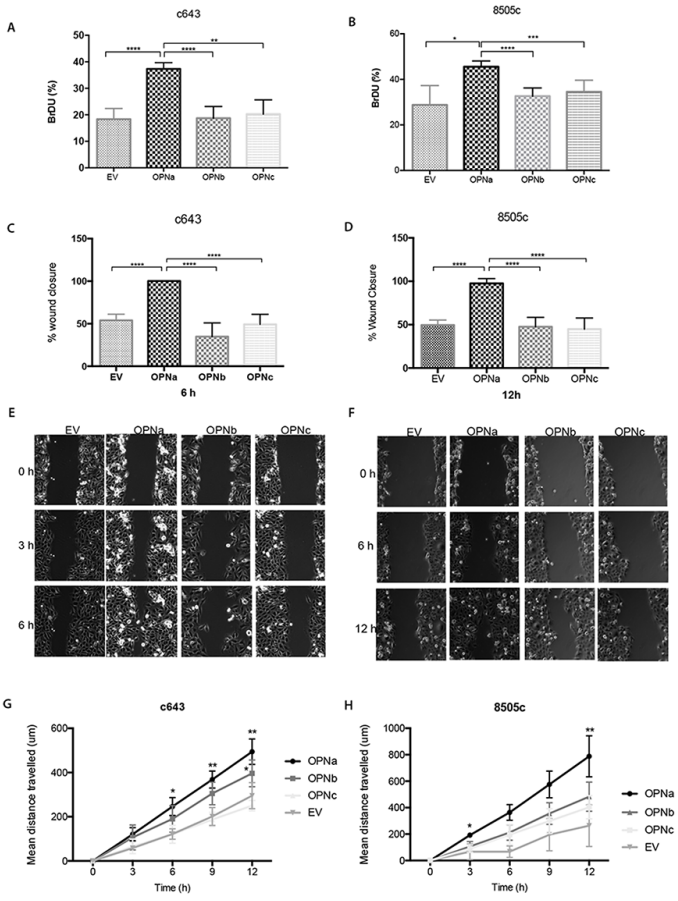

Figure 4: Cells overexpressing OPNa stimulate cell growth, migration and motility. DNA synthesis and proliferation rates have been evaluated by BrdU incorporation assays; **A.** c643 and **B.** 8505c cell clones (overexpressing EV, OPNa, OPNb, OPNc, respectively). Effect of each OPN-SV overexpression in c643 **C.** and in 8505c **D.** cells on migration rates using wound-healing assays. Representative images are shown for cell migration in c643 **E.** and 8505c **F.** OPN-SV overexpressing cells, which were monitored by time-lapse microscopy for 0, 3, 6, 9 and 12 hours after cell scratch. The % wound closure in the graphs represents the wound measured area. For motility assays, OPNa, OPNb, OPNc and EV cell clones were cultured in µ-Slide 4 Well $^{Ph+}$ and monitored by time-lapse microscopy for 12 hours. Four microscope fields were averaged for each c643 **G.** and of 8505c **H.** OPN-SV overexpression clones and controls containing 10 cells/field (Representative videos are available in Online Supplemental Material). Graph data corresponds to motility rates from two independent experiments and values are expressed as mean ± se. *p < 0,05; ** p < 0,01; **** p < 0,0001. All the experiments were done in triplicated.

circumscribed fvPTC. In this last point it is also worth to mention that our results in OPN expression fits with the recent proposal for the reclassification of encapsulated fvPTC as NIFT ("noninvasive follicular thyroid neoplasm with papillary-like nuclear features") due to the very low risk of adverse outcome of these patients [27].

Further, we found that high levels of tOPN expression in cPTC are associated with increased tumor size, presence of extrathyroid extension, vascular invasion and *BRAF*^{V600E} mutation. Our results regarding tOPN are in accordance with previous studies demonstrating tOPN overexpression in PTC samples [15]. Our results also corroborates previous studies which showed that tOPN overexpression (transcript and/or protein) are significantly associated with poor prognostic factors, such as presence of lymph node metastasis, tumor size and poor disease free survival in PTC samples [13, 14, 16, 17].

We also observed that tOPN protein overexpression in cPTC is significantly associated with hyaline stroma. These data are in accordance with OPN as a glycoprotein secreted in the extracellular matrix, both in tumor and non-

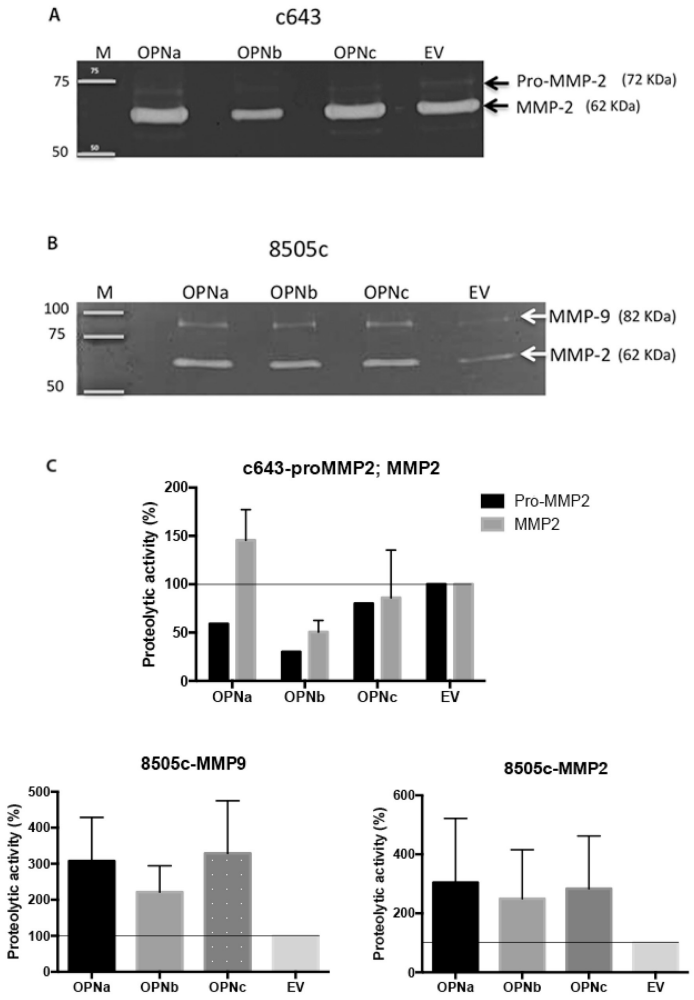

Figure 5: MMP2 and MMP9 activity in cells overexpressing OPN-SV. Gelatin zymography assay was used to analyze the levels of active MMP2 and MMP9 in conditioned medium from **A.** c643 (overexpressing OPNa, OPNb, OPNc and EV) and from **B.** 8505c (overexpressing OPNa, OPNb, OPNc and EV) cell clones. Representative images are shown. On the left are depicted molecular weights of standard markers; on the right are shown the variants of metalloproteinases (MMPs) observed in the gels. **C.** The intensity of gelatin-digested bands by pro- MMP2, MMP2 and MMP9 were measured by densitometry and are represented by the diagram bar. Percentage (%) of proteolytic activity from 8505c and c643 cells overexpressing OPNa, OPNb or OPNc was compared with the activity present in culture medium from 8505c and c643 control cells (EV). Data correspond to mean values of two independent experiments.

tumoral tissues [28]. OPN is also known to be upregulated in the tissue stroma in different conditions, such as salivary pleomorphic adenomas [29] and intrahepatic cholangiocarcinoma [30]. It is well known that the interplay between epithelial cells and the microenvironment may contribute to keep the epithelial polarity and to modulate growth inhibition [31]. On the other hand, the stromal compartments undergo changes in response to emerging epithelial lesions that can have a key role in cancer initiation and progression [31, 32]. Accordingly, our observed data regarding the correlation of tOPN and the presence of a hyaline stroma support the assumption that either tOPN expression alone or tOPN in association to hyaline stroma may play a role in cPTC tumor aggressiveness.

Our data show that OPNa variant is overexpressed and specifically associated with poor prognostic features in PTC. In particular, OPNa variant was significantly associated with presence of extrathyroid extension, vascular invasion and $BRAF^{V600E}$ mutation. In contrast to this, OPNb was only significantly associated with $BRAF^{V600E}$ mutation. In the fvPTC, the only significant association observed was between high tOPN and OPNa expression levels and older patients. Based on these findings, we postulate that elevated OPNa expression in TC may occur during tumor progression, facilitating more aggressive phenotypes. Some authors have shown that OPN-SV expression and its associations to clinicopathological features seem to be tissue specific. For instance, it has been reported that high OPNb and OPNc expression levels in breast cancer [33] and in gastric tumors [34] is correlated with more aggressive clinicopathological features. In a combined expression analysis, OPNc, ER and HER2 can reliably predict grade 2-3 in breast cancer samples [35]. Moreover, in a prostate tumor model, our group has also observed that OPNc overexpression is correlated to poor prognostic features [36]. Conversely, our data show that OPNa has a more relevant prognostic role in PTC than the other OPN-SV.

To assess the possible impact of OPN-SV in TC cell properties we ectopically overexpressed OPNa, OPNb or OPNc in c643 and 8505c cell lines with plasmid constructs containing each of the three OPN-SV. We observed that TC cells overexpressing OPNa displayed significantly increased cell growth, migration and motility, whereas OPNb and OPNc overexpression in TC cells did not induce similar effects. Other reports have demonstrated that OPN-SV play an important role in tumor progression by regulating cell growth, adhesion, migration and tumor formation. As reported by Lin J and co-workers [37], OPNb overexpressing cells derived from esophageal adenocarcinoma also evoked enhanced cell proliferation, migration and invasion. Additionally, our

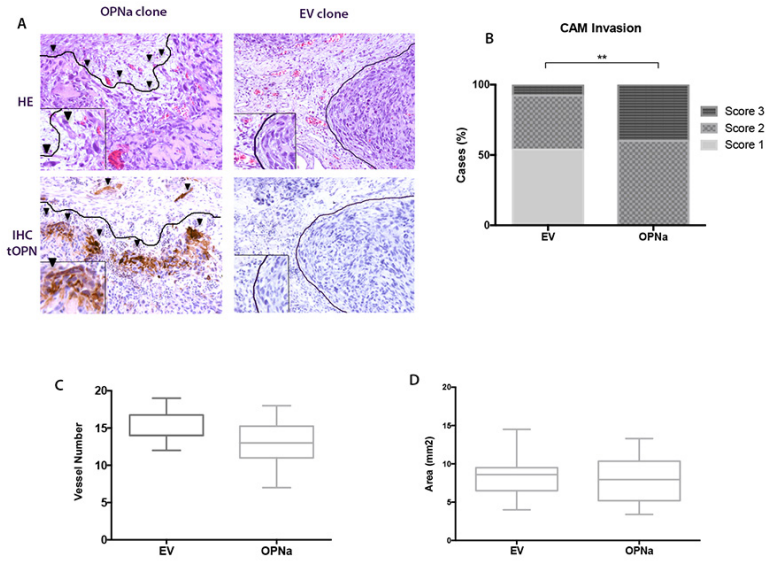

Figure 6: Overexpression of OPNa increases the invasive potential of TC cells in the CAM assay. A. Invasive capacity of inoculated cells was evaluated by HE and IHC using an anti-tOPN antibody in xenograft CAM tumor sections. Positive human tOPN immunostaining demonstrating effective overexpression of OPN in CAM tumors originated from OPNa clones. c643-EV cells forming compacted tumors (score 1; full lines surrounding the tumor bulk, right panels). OPNa tumors exhibiting loosen structures and single cells invading the xenograft CAM tumor mesenchyme (arrow heads; score 3, left panels); **B.** Invasion score analysis demonstrating c643-OPNa overexpressing cells and their corresponding invasive score, when compared to EV control clones ($p = 0.003$) **C.** CAM angiogenic assay, as demonstrated by the number of formed vessels in c643-OPNa and EV control clones **D.** CAM xenograft tumors areas measured in mm^2 indicating tumorigenesis in c643-OPNa and EV clones.

group also previously demonstrated that OPNc activates invasion and adhesion properties, as well as metastatic potential and angiogenesis in a prostate and an ovarian carcinoma model [26, 38]. In prostate cancer cells, OPNc can activate AR signaling [39] and resistance to docetaxel [40]. Furthermore, other groups demonstrated that in hepatocellular carcinoma cells, OPNa and OPNb can induce cell migration [25]. Our findings highlight the importance of OPNa in TC cell growth and migratory and invasive phenotype.

To further explore OPNa roles on modulating TC invasive properties, we also explored the role of OPNa in the activation of matrix metalloproteinases (MMPs). MMPs are important enzymes in the metastatic cell arsenal. These proteins can degrade both cell adhesion molecules and extracellular matrix molecules, enabling tumor cells both to migrate from the tumor bulk and to invade adjacent tissues [41]. Previous data from our group in prostate and ovarian tumor models showed that cells overexpressing the OPNc variant induce expression of MMP2 and MMP9, highlighting the functional tissue specificity of OPN-SV [38]. Thyroid carcinomas produce elevated levels of MMP2, which has been correlated with the presence of lymph node metastasis [51]. Herein, we have found that CM collected from cells overexpressing OPNa have increased activity of MMP2 in c643 cells and MMP2 and MMP9 in 8505c cell lines. These results evidence that OPNa may promote TC cell invasion through inducing MMP2 and MMP9 secretion. The detailed mechanism by which this event occurs still needs further characterization. Nonetheless, it has been described that OPN can regulate MMPs activity by binding to pro-MMP9, thus promoting its activation [42]. Additionally, OPN can induce NFkB-mediated pro-MMP2 and MMP9 activation through IkBa/IKK signaling pathway [43].

Since MMPs expression and cancer cell migration are fundamental features for tumor invasion [44], we further investigated the contribution of OPNa variant for a TC cell line invasiveness using an *in vivo* experimental model. Using the CAM assay approach, we observed that tumors formed by these cell clones present a loosen structure, in which the cells were oriented towards the invasion front while single cells and cell clusters invading the CAM mesenchyme could also be observed. In contrast to this, EV clones formed compact tumors, with clear defined boarders lacking invading cells. Similarly to our data, overexpression of OPNa has been previously associated with the cancer cell invasion modulation in mesothelioma, breast cancer and hepatocellular carcinomas [25, 45, 46]. Although activating cell invasion, OPNa overexpression does not significantly modulated angiogenesis and tumorigenesis in this *in vivo* tumor model. Some oncogenic proteins can activate some specific steps in tumor progression, but not others. As evidenced by our data OPNa overexpression *in vivo* may predominantly modulate signaling pathways that stimulate migration and invasion, possibly through stimulating extracellular matrix degradation by MMP2 and MMP9. In light of our results, we may hypothesize that the increased invasive capacity of c643 cells overexpressing OPNa (in comparison to c643-EV cells) allows the cells to reach the blood vessels, and have no effect in the recruitment of new vessels.

In conclusion, our data demonstrated that OPNa is the prevalent OPN-SV in DTC tissues and cell lines, and that overexpression of OPNa is associated with poor prognostic and invasive features in cPTC. Moreover, OPNa overexpression in TC cell lines strongly increases cell migration, invasion and MMPs activity, evidencing a major role for OPNa in TC progression features. Taken together, these features provide early evidence that OPNa can potentially mediate invasive and metastatic potential in cPTCs.

MATERIALS AND METHODS

Tumor specimens

We evaluated adjacent thyroid tissues (n = 20), thyroid adenomas (n = 6), follicular thyroid carcinoma (FTC) (n = 12), follicular variant of papillary thyroid carcinoma (fvPTC) (n = 22) and classic papillary thyroid carcinoma (cPTC) (n = 69). All the analyzed specimens were collected from primary tumors, surgically resected at the Centro Hospitalar São João, Porto, Portugal. After surgery, samples were immediately snap-frozen and stored at -80°C until use. Additional fragments were fixed in 10% buffered formalin and embedded in paraffin (FFPE). The histologic diagnosis of all cases were reviewed by three thyroid pathologists (CE, ER, MSS) according to the WHO classification [47]. Clinicopathological and molecular features are summarized in Supplementary Table S2. All the procedures described in this study were approved by the respective ethical boards and are in accordance with national and institutional standards.

Immunohistochemistry

OPN IHC analysis was performed in representative tumor tissue sections of 44 cPTC, 16 fvPTC and 10 FTC samples using an antibody that recognize all three OPN-SV (anti-total OPN- tOPN) (polyclonal, goat, 1:500, R&D Systems). Normal gallbladder was used as a positive control, once it has been previously reported to overexpress tOPN [48, 49]. IHC procedure was done according to [49]. Semi-quantitative IHC evaluation was independently performed by two observers (CE and LBF). Total OPN staining was scored in the range 0-7, which corresponds to the sum of the staining intensity (absent = 0, faint = 1, moderate = 2 and strong = 3) plus the proportion of positively stained cells (<5% = 0; 5-25% = 1; 25–50% = 2, 50–75% = 3 and >75% = 4) (Table 1).

RNA extraction, reverse transcription and real time PCR

Total RNA was extracted from cell lines and tumor tissues using Trizol reagent (Life Technologies, GIBCO BRL). For cDNA preparation, 1 μg of total RNA was reverse transcribed using the RevertAid first-strand cDNA synthesis kit (Fermentas, Burlington, ON, Canada).

Each OPN-SV transcript region was amplified with specific oligonucleotide pairs (Supplementary Table S3 and Supplementary Figure S1A). Quantitative PCR reactions were conducted using SYBR Green detection system (Applied Biosystems, Warrington WA1 4SR, UK). Conditions for OPN-SV amplification were 50º C for 2 minutes, 94º C for 5 minutes followed by 40 cycles of 94º C for 30 seconds, 60º C for 30 seconds, and 72º C for 45 seconds. Relative gene expression was calculated using the Delta-Delta CT method. GAPDH gene was used as the constitutive control.

Cell culture, OPN plasmids and transfections

We analyzed eight TC cell lines: TPC1, KAT4, Hth74, XTC1, 8505c, K1, BCPAP and c643, from which two were selected for stable transfection. All cell lines were authenticated using DNA profile analysis, obtained with the PowerPlex 16 system (Promega, Madison, USA), according to ATCC and HSRRB available DNA profiles [50]. All the cell lines were cultured in standard culture medium, supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin and 100 mg/ml streptomycin in a humidified environment containing 5% CO_2 at 37˚C. The open reading frame of OPN-SV, was cloned into pCR3.1 mammalian expression vector, as previously described [51]. OPN-SV expression vectors (kindly provided by Dr. George Weber (Cincinnati University)) were used to transfect c643 and 8505c cell lines (the ones with lower OPN expression; Figure 2F) in order to overexpress each OPN-SV. These cells were also transfected with the pCR3.1 control empty vector (EV). Transfections were performed using LipofectamineTM 2000 (Invitrogen, CA). Expression plasmids were transfected into c643 and 8505c cells and the stably overexpressing cell clones were selected using 600 μg/ml of G418 for c643 and 800 μg/ml for 8505c cell lines.

Immunocytochemistry

Cells plated on coverslips were fixed in 4% paraformaldehyde for 20 min at room temperature (RT). Cells were emerged in NH_4Cl 50 mM in PBS during 10 min, and then, cells were permeabilized in 0.2% Triton X-100 and blocked in 5% BSA in PBS for 30 min at RT. Primary antibodies were diluted in PBS containing 5% BSA and incubated overnight at 4ºC as follows: rabbit polyclonal against OPN (Rockland, Limerick, PA, USA, diluted 1:500). Coverslips were washed in 0.1% Triton X-100 prepared in PBS (PBT) and incubated with goat anti-rabbit IgG secondary antibodies conjugated with Alexa Fluor 594 (Invitrogen; diluted 1:300 in 5% BSA–PBT) for 1h at RT. Nuclei were stained with 0.1 mg/ml diamino phenylindole (DAPI; Sigma–Aldrich). Images were taken by a Zeiss fluorescence microscope with ApoTome attachment (Axio Imager Z1 stand).

Cell proliferation

Proliferation was measured by evaluating bromodeoxy uridine (BrdU) incorporation in c643 and 8505c OPN-SV transfected cells as previously described [52]. Quantification of BrdU positive cells was performed using ImageJ software, by counting the percentage of BrdU-positive nuclei on a total of 1500 cells.

Cell migration and motility assays

For cell migration assays, c643 and 8505c (overexpressing OPNa, OPNb, OPNc or EV) clones were seeded until forming a confluent monolayer. The cell wound was created by scraping the cell monolayer with a pipette tip. After scratch, cell migration was monitored by time-lapse microscopy and images were taken every 10 minutes for 24 hours.

For motility assays, 3,0 x 10^5 cells of c643 and 8505c OPNa, OPNb, OPNc or EV transfected cells were plated in μ-Slide 4 Well Ph+ and monitored by time-lapse video microscopy to evaluate cell motility. Migratory tracks were measured for individual cells overexpressing each OPN-SV or EV cells using ImageJ software. Four microscope fields containing around 10 cells per field were monitored for each cell line.

Gelatin zymography

2.5 x 10^5 cells from c643 and 8505c (overexpressing OPNa, OPNb, OPNc or EV) were seeded to generate conditioned medium (CM). Matrix metalloproteases (MMPs), namely MMP2 and MMP9, activity was assessed in the CM of each condition by gelatin zymography, as previously described [53].

In vivo chicken embryo chorioallantoic membrane (CAM) angiogenesis, tumorigenesis and invasion assays

In vivo angiogenic activity of c643 cells overexpressing OPNa and the EV controls were assessed by chicken chorioallantoic membrane (CAM) assay, as previously described [54]. According to the European Directive 2010/63/EU, ethical approval is not required for experiments using embryonic chicken. Correspondingly, the Portuguese law on animal welfare does not restrict the use of chicken eggs.

CAMs bearing the tumors were fixed in 10% neutral-buffered formalin and paraffin-embedded for slide sections. Sections were HE stained for histological examination or processed for anti-tOPN IHC analysis to validate OPN overexpression in CAM-xenografted tumor cells. These xenograft tissue sections were also used to evaluate cell invasion. The analysis was performed in a blind fashion manner by two independent observers and slides were scored as follows: score 1- Tumor cells are tight together forming a compact mass. The invasion front (area where tumor cells touch the CAM mesenchyme) is clearly defined as an encapsulated -like structure; score 2- Tumor cells are more loosen at the core of the tumor and in some cases, matrigel can be detected. Cells are oriented towards the invasion front; score 3- Tumor cells are oriented towards the invasion front and it is possible to observe single cells or small clusters of cells disconnected from the invasive front.

Data and statistical analysis

Statistical analyses were performed using 22.0 SPSS statistical package (IBM, 2014). X^2 and independent samples t-test were performed to verify if there was any association(s) between OPN expression and clinicopathological data. GraphPad was used for the construction of the graphs. ANOVA test was used to calculate significance in the CAM angiogenic and tumorigenic assays. A ChiSquare test was used to calculate significance in the CAM invasion assay. All results are presented as mean ± standard error. Values of $p \leq 0.05$ were considered to be statistically significant.

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CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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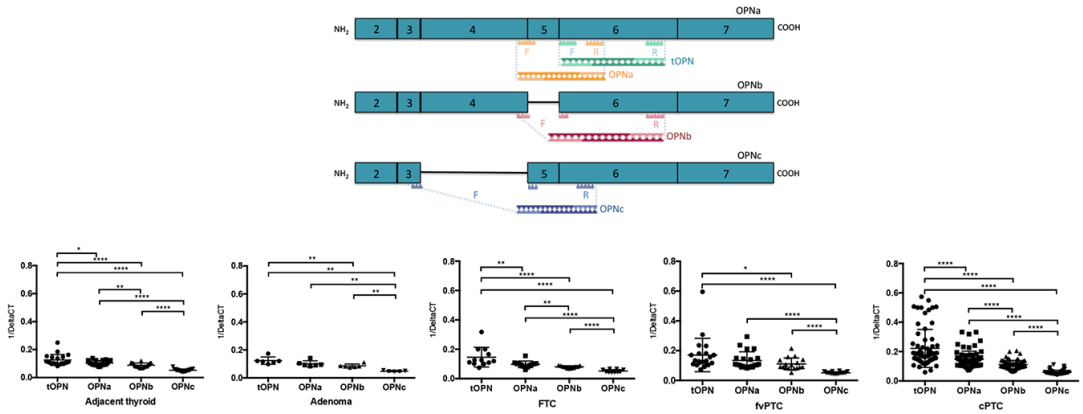
Osteopontin-a splice variant is overexpressed in papillary thyroid carcinoma and modulates invasive behavior

SUPPLEMENTARY FIGURE, TABLES AND VIDEOS



Supplementary Figure S1: Schematic representation of three OPN splice variants (OPNa, OPNb and OPNc). Figure depicts oligonucleotide primers annealing sites for amplification of each specific OPN splice variant and tOPN. OPNa (full-length splice variant), OPNb (lacks exon 5) and OPNc (lacks exon 4). Green: primers used for tOPN amplification (forward and reverse primers, located at exon 6) and the corresponding green tOPN amplification product; Orange: primers used for OPNa amplification (forward primer located at exon 4-5 splice junction; reverse primer located at exon 6) and the corresponding OPNa orange amplification product; Red: primers used for OPNb amplification (forward primer, located at exon 4-6 splice junction; reverse primer, located at exon 6) and the corresponding red OPNb amplification product; Blue: primers used for OPNc amplification (forward primer – exon 3-5 junction; reverse primer - exon 6) and the corresponding blue OPNc amplification product. Numbers in the boxes represent the coding exons. **B.** OPN-SV transcript expression levels (symbols: tOPN -•; OPNa -■; OPNb -▲, OPNc -▼) in adjacent thyroid, follicular adenomas, FTC, fvPTC and cPTC samples.

Supplementary Table S1: Analyses of total OPN (tOPN) IHC staining in well and poorly circumscribed fvPTC samples

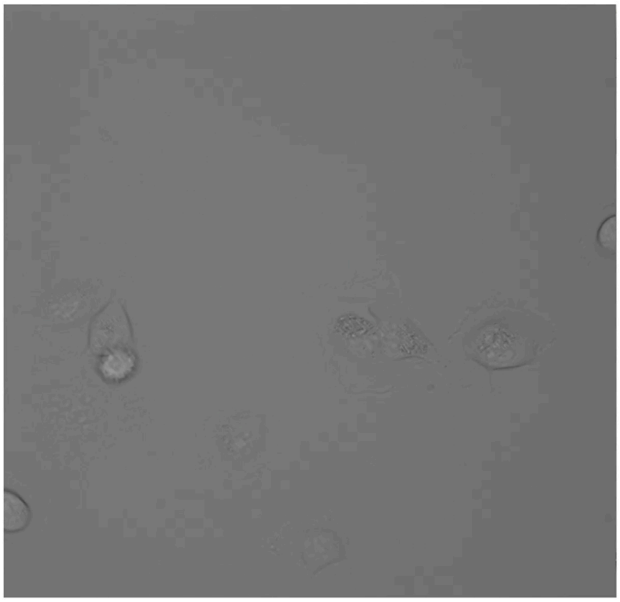
fvPTC	N	Score Mean	
Well circumscribed	13 (81.25%)	1	
Poorly circumscribed	3 (18.75 %)	2.33	***p=0.27***
Total	16 (100%)		

Supplementary Table S2: Summary of the clinical, pathological and molecular data of the FTC, fvPTC and cPTC cases

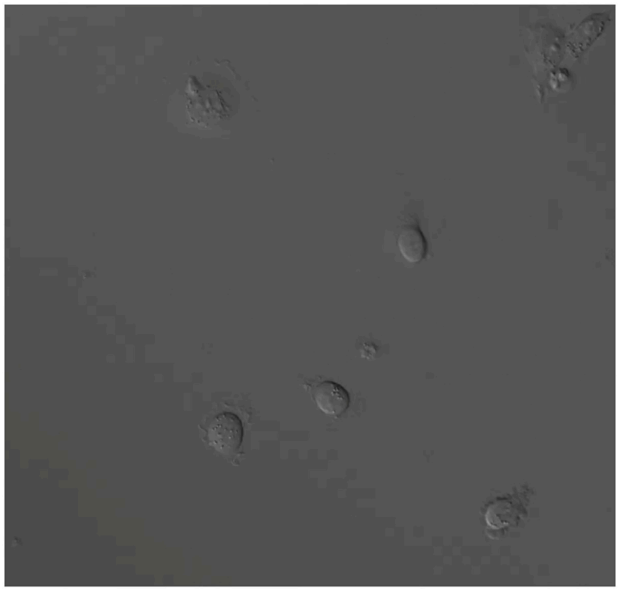
Variable	FTC	fvPTC	cPTC
Gender			
Female	4 (66.7%)	20 (90.1%)	54 (84.4%)
Male	2 (33.3%)	2 (9%)	10 (15.6%)
Age (yr) (mean ±S.D.)	54.7 (±15.8)	40.1 (±13.1)	41.5 (±15.4)
Stroma			
Absent	-	-	17 (47.2%)
Present	-	-	19 (52.8%)
Tumor size (cm)			
(mean ±S.D.)	4.1 (±1.2)	2.6 (±1.5)	2.6 (±1.4)
Extrathyroid Extension			
Absent	-	10 (83.3%)	24 (51%)
Present	-	2 (16.7%)	23 (49%)
Invasion (vascular and/or capsular)			
Absent	0	13 (68.4%)	24 (42.8%)
Present	6 (100%)	6 (31.6%)	32 (57.2%)
Lymph Node Metastasis			
Absent	-	12 (75%)	25 (53.2%)
Present	-	4 (25%)	22 (46.8%)
Thyroiditis			
Absent	-	-	2 (20%)
Present	-	-	8 (80%)
***RET/PTC1* translocation**			
Absent	11 (100%)	21 (95.5%)	45 (83.3%)
Present	0	1 (4.5%)	9 (16.7%)
***BRAF*^{V600E} mutation**			
Absent (n=26)	11 (100%)	20 (91%)	25 (43.1%)
Present (n=30)	0	2 (9%)	33 (56.9%)
***TERT* mutation**			
Absent	7 (87.5%)	22 (100%)	51 (96.2%)
Present	1 (12.5%)	0	2 (3.8%)
***RAS* mutation**			
Absent	11 (91.7%)	18 (81.8%)	50 (92.6%)
Present	1 (8.3%)	4 (18.2%)	4 (7.4%)

Supplementary Table S3: Forward and reverse oligonucleotide sequences used for tOPN, OPN-SV and GAPDH specific amplification

Gene	Oligonucleotide Name	Sequence 5' – 3'
tOPN	tOPNF	CCA ACG AAA GCC ATG ACC AC
	tOPNR	CTG TGG GGA CAA CTG GAG TG
OPNa	OPNaF	ATC TCC TAG CCC CAC AGA AT
	OPNaR	CAT CAG ACT GGT GAG AAT CAT C
OPNb	OPNbF	CTC CTA GCC CCA CAG ACC CT
	OPNbR	TAT CAC CTC GGC CAT CAT ATG
OPNc	OPNcF	CTG AGG AAA AGC AGA ATG
	OPNcR	AAT GGA GTC CTG GCT GT
GAPDH	GAPDH-F	TGA CCC CTT CAT TGA CCT CA
	GAPDH-R	AGT CCT TCC ACG ATA CCA AA



Supplementary Video S1: Motility 8505c-OPNa: Representative video of motility assay used to evaluate the motility of 8505c cells overexpressing OPNa splice variant.



Supplementary Video S2: Motility 8505c-EV: Representative video of motility assay used to evaluate the motility of 8505c cells overexpressing empty vector (EV) control.

Chapter 4 – Paper II - OPNa expression is associated with the formation of psammoma bodies in papillary thyroid cancer: *in vivo* and *in vitro* evidence

This chapter is presently a manuscript in preparation with the same title

Title: **OPNa variant expression is associated with calcification and formation of psammoma bodies in papillary thyroid carcinoma: *in vivo* and *in vitro* evidence**

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Keywords: Osteopontin splice variants (OPN-SV), osteopontin-a (OPNa), thyroid cancer, psammoma bodies, calcification

ABSTRACT

Background: Osteopontin (OPN) and its three spliced variants (OPN-SV: OPNa, OPNb and OPNc) are overexpressed in several tumours and frequently associated with cancer progression. This holds true for papillary thyroid carcinoma (PTC) which is the most common variety of thyroid cancer (TC) being the histologic type which often presents desmoplasia (collagen deposition) and dystrophic calcification, including a fairly typical features, the psammoma bodies (PB).

Objective: The aim of this study was to investigate the role of OPN-SV expression in the development of PB in classical variant of PTC (cPTC).

Methods: Total OPN and OPN-SV expression was analysed by immunohistochemistry and real time PCR in a series of 48 cPTC cases and three diffuse sclerosing PTCs. The association of OPN expression and the presence of PB as well as between PB in cPTC and the clinicopathological features of the tumours were evaluated. TPC-1 and c643 TC cell lines overexpressing OPN-SV were tested for the ability to promote calcification and to synthesize collagen *in vitro*.

Results: Overexpression of OPNa transcripts was significantly associated with the presence of PB in cPTC samples. The presence of PB in cPTC was associated with younger patients and lymph node metastasis. Moreover, OPNa overexpression displayed a strong capacity to promote calcification and substantial collagen synthesis in the thyroid cancer cell lines.

Conclusion: Our data suggest that OPNa plays a role in the formation of PB often associated with cPTC. Basic research on the interactions between OPNa overexpression

by tumour cells and the surrounding microenvironment can give clues for a better understanding of cPTC biology and phenotype.

4.1 Introduction

Thyroid cancer (TC) is the most prevalent endocrine malignancy, being papillary thyroid carcinoma (PTC) the most common histologic type, accounting for approximately 80% – 90% of human TCs [1, 2]. The rising incidence of TC is almost entirely attributed to the increased incidence of PTC cases [2, 3]. PTC display genetic alterations such as $BRAF^{V600E}$ mutations, *RET/PTC* rearrangements and *RAS* mutations. However, the etiopathogenesis of these carcinomas is not completely understood [4].

Previous reports have shown that clinicopathological features, such as patient age, sex, tumour size, histological grouping, extrathyroid extension and lymph node status are useful prognostic factors in PTC patients [5-7]. Calcification, which is a frequent histological feature in several cancers, is not generally valued in PTC, although being usually detected by ultrasonography in the preoperative evaluation of thyroid nodules [8, 9]. Despite the fact that calcification may be present in both benign and malignant thyroid lesions, its association with malignancy has been consistently verified in a few reports [10, 11].

Based on histological characteristics, TC calcification can consist in dystrophic calcification or in formation of psammoma bodies (PB). PB are defined as spherical (50–70 mm round) calcified foci with concentric laminations, presenting a glassy appearance [12-14]. The genesis of PB in PTC is not completely understood. Some authors proposed that PB may be formed by (i) thickening of the base lamina of the vascular stalk of the neoplastic papillae, followed by vascular thrombosis, calcification, and tumour cell necrosis and/or (ii) necrosis and calcification in intralymphatic tumour thrombi in the thyroid adjacent to the tumour or in the opposite thyroid lobe [13]. The presence of PB is easily detected in cytological or histologic specimens. In fine-needle aspiration (FNA)

biopsies, it has been correlated to malignancy, and to PTC in particular [10]. In PTC, PB are almost exclusively observed in classical variants of PTC (cPTC) but also in some other variants of PTC (such as diffuse sclerosing variant and tall cell) [15], and have been considered as a feature that supports PCT diagnosis [10, 12, 15].

OPN is recognised as a multifunctional phosphoglycoprotein, with roles which include bone remodelling and mineralization. In normal bone tissue, OPN is expressed by osteoclasts and osteoblasts, which are responsible for bone remodelling. This protein has calcium-binding properties and hydroxyapatite affinity. It has been proposed that the OPN phosphate groups steric arrangements are needed for calcium binding and further apatite crystal formation during bone matrix mineralization [16].

Earlier reports showed that total OPN (tOPN) overexpression seems to plays a role on the formation of PB in PTC samples. Tunio *et al*. [17] observed that OPN overexpression in PTC cells was found around PB and that OPN transcript-expressing cells were identified as CD68-positive macrophages. In a more recent study, OPN expression in PTC samples was significantly associated with the presence of PB [18].

Recently, we demonstrated that among the different spliced variant of osteopontin (OPN-SV) primary transcripts, OPNa (which contains the full length coding sequence) is overexpressed in cPTC samples. This overexpression was associated with aggressive clinicopathological features of PTC. Additionally, we showed that OPNa overexpression induced cell proliferation, migration, motility and invasion rates in TC cell lines [19].

Despite the aforementioned studies, little is known about the molecular interactions that result from PB in cPTC and OPN overexpression and the relative relevance of the different OPN-SV in the process. The goal of this study was to evaluate the putative associations between the expression of tOPN and its variants with PB and the involvement of OPN on PTC calcification.

4.2 Material and Methods

Tumour specimens

Tissue specimens were collected from primary tumours, surgically resected at the Centro Hospitalar São João (CHSJ), Porto, Portugal. This series is composed by three cases of diffuse sclerosing variant of PTC and 48 cases of cPTC. After surgery, samples were immediately snap-frozen and stored at -80°C until use. Additional fragments were fixed in 10% buffered formalin and embedded in paraffin (FFPE). The histologic diagnosis of all cases were reviewed by pathologists specialized in thyroid cancer according to the WHO classification criteria [20]. Clinicopathological and molecular features are summarized in Table 1. All the procedures described in this study are in accordance with national and institutional ethical standards.

Cell culture

The expression levels of tOPN and OPN-SV have been established in TC cell lines in a previous study from our group [19]. For this study, the TPC1 and c643 cell lines (presenting respectively the highest and lowest tOPN and OPN-SV levels) have been selected for experimental approaches.

c643 cells stably overexpressing OPN-SV were used, as described in Ferreira LB *et al*. [19]. Briefly, the open reading frame of OPNa, OPNb and OPNc were cloned into pCR3.1 mammalian expression vector as previously described by He B *et al*. [21]. These plasmid constructs were used for transfection of c643 cells. Transfections were carried

out using Lipofectamine 2000 (Invitrogen). The OPN variants/pCR3.1 plasmids or the vector alone (Empty Vector) were transfected into c643 cells and the stably expressing cells were selected with 600 μg/mL of G418 in the culture medium. All the cell lines were cultured in RPMI 1640 cell culture medium with ultraglutamine, supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin and 100 mg/ml streptomycin in a humidified environment containing 5% CO_2 at 37˚C.

Immunohistochemistry

OPN immunohistochemistry (IHC) analysis was performed in representative tumour tissue sections using an anti-total OPN antibody (anti- tOPN) (polyclonal, goat, 1:500, R&D Systems) that recognize all three OPN-SV. Normal gallbladder tissue samples previously reported to overexpress tOPN [22], was used as a positive control. IHC procedures were done according to Ferreira LB *et al*. [22]. Semi-quantitative IHC evaluation was independently performed by two observers (CE and LBF). Total OPN staining was scored in the range 0-7, corresponding to the sum of the staining intensity (absent = 0, faint = 1, moderate = 2 and strong = 3) plus the proportion of positively stained cells (<5% = 0; 5-25% = 1; 25–50% = 2, 50–75% = 3 and >75% = 4).

RNA extraction, reverse transcription and Real time PCR

Total RNA was extracted from cell lines and tumour tissues using Trizol reagent (Life Technologies, GIBCO BRL). For cDNA preparation, 1 μg of total RNA was reverse transcribed using the RevertAid first-strand cDNA synthesis kit (Fermentas, Burlington, ON, Canada).

Real time PCR analysis was carried out for each OPN-SV transcript region with a SYBR Green detection system (Applied Biosystems, Warrington WA1 4SR, UK) using the previously described primers [19] and the following conditions: 50º C for 2 minutes, 94º C for 5 minutes followed by 40 cycles of 94º C for 30 seconds, 60º C for 30 seconds, and 72º C for 45 seconds. Relative gene expression was calculated using the Delta-Delta CT method. GAPDH gene was used as the housekeeping control.

Matrix mineralisation assessment

In order to evaluate matrix calcification, c643 overexpressing OPN-SV and TPC1 cell lines were cultured in 24 well plates (1.5 x10^5 cells/well) for 31 days. Following 10, 17, 24 or 31 days in culture, cells were fixed with 4% paraformaldehyde (PFA) in PBS and evaluated for calcium deposits production by staining with 1 % Alizarin Red solution in 2% ethanol. Cells were then washed with water three times and representative images for each culture condition were captured with NIKON microscope and NIKON Digital DLS Camera.

Matrix collagen production assay

c643 overexpressing OPN-SV and TPC1 cell lines were cultured in 24 well plates (1.5 x10^5 cells/well) for 31 days. Following 10, 17, 24 or 31 days in culture, cells were fixed with 4% paraformaldehyde (PFA) in PBS and evaluated for collagen fibbers production by Masson's trichrome staining. Briefly, cells were immersed in Richard-Allan Scientific® Bouin's Fluid (Thermo Scientific) solution for 5 minutes at room temperature, washed with water followed by incubation in Celestin Blue (Thermo

Scientific) for 6 minutes and then in Gil's hematoxylin staining solution (Sigma-Aldrich, St. Louis, MO) for 5 min. Cells were washed with 1% acid alcohol, three times and with water for further three times. Cells were then immersed in Biebrich scarlet-acid fuchsin (Thermo Scientific) for 5 min, placed in phosphotungstic and phosphomolybdic acid solution (Thermo Scientific) for 5 min, moved to Aniline Blue solution (Thermo Scientific) for 5 min and then placed in 1 % acetic acid solution for 2 min. Finally, cells were rinsed in deionized water and representative images for each culture condition were captured with NIKON microscope and NIKON Digital DLS Camera.

Statistical analysis

Statistical analysis was performed using 22.0 SPSS statistical package (IBM, 2014). Independent-samples *t*-test test was performed to verify if there were any association(s) between OPN expression and the presence of PB. The significance of any correlation between the presence of PB and the clinicopathological features was determined by either the X^2 test or Fisher's exact test (two-sided). The predictive value of PB and other factors (sex, age, presence of stroma, tumour size, absence of capsule, extrathyroid extension, invasion, lymph node metastases, *RET/PTC* rearrangement, $BRAF^{V600E}$ and *RAS* mutations) were assessed using univariated and multivariated logistic regression models. For *in vitro* experiments, data are represented as mean ± standard error and analysed using Mann-Whitney test. Values of $p < 0.05$ were considered statistically significant.

4.3 Results

PB are strongly stained by anti-tOPN in cPTC samples

IHC staining for tOPN was performed in three cases of diffuse sclerosing PTC samples and 48 cases of cPTC, following a score criteria based on our previous report [22]. Total OPN expression was observed in 60.4% of the cPTC cases (29 out of 48). The three diffuse sclerosing PTC samples were virtually negative for tOPN staining. (Table 2). The samples were also carefully analysed for the presence of PB according to HE and anti-tOPN antibody staining. As shown in Figure 1, right panel, PB were strongly stained by tOPN antibody, allowing a much clearer view of PB in the cPTC cases, when compared to HE staining. PB were present in 53% (24 out of 45) of the cPTC cases according to anti-tOPN staining. No statistically significant association was found between the presence of PB and tOPN expression ($p = 0.71$) (Table 3). Similarly, no association was found between tOPN protein expression and sex, age, tumour size, extrathyroid extension, lymphovascular and/or capsular invasion, lymph node metastases, *RET/PTC* rearrangements, $BRAF^{V600E}$ or *RAS* mutations. On the other hand, high levels of tOPN protein expression was associated with the presence of stroma in cPTC samples ($p = 0.01$) (Table 4).

The presence of PB is correlated with cPTC clinicopathological features

We evaluated the correlation between the presence of PB and cPTC clinicopatological features. An association was found between the presence of PB and cPTC in younger patients ($p = 0.005$) and those presenting lymph node metastases ($p =$

0.03) (Table 5). No significant associations were observed between the occurrence of PB and patients' sex, stroma, tumour size, extrathyroid extension, lymphovascular and/or capsular invasion, *RET/PTC* translocation, $BRAF^{V600E}$ and *RAS* mutations.

Regression model was performed to evaluate factors associated with lymph node metastases in cPTCs (Table 6). A total of 14 patients (40%) had lymph node metastases. Younger age (odds ratio (OR) 4.4; p = 0.05) and the presence of PB (OR 7.3; p = 0.02) were associated with lymph node metastases. However, when introducing features significantly associated with lymph node metastases (using the univariate model) in the multivariate regression analysis, only PB was found to be significant (OR 5.6; p= 0.05).

OPNa transcript overexpression is associated with presence of PB in cPTC samples

Since we found a higher tOPN protein expression in cPTC samples containing PB (although not attaining statistically significant association (Table 3)), we then evaluated transcript expression levels of each OPN-SV (OPNa, OPNb or OPNc) and of tOPN to analyse their relative contributions to PB formation. Of note, tOPN expression corresponds to the sum of all OPN-SV. In this study, we have found that cPTC cases that present PB also have higher tOPN, OPNa, OPNb and OPNc transcript levels than those cases lacking PB. Interestingly, only OPNa and OPNb levels were significantly higher ($p < 0.05$) (Figure 2 and Table 7). In the three diffuse sclerosing PTC cases, we found that OPNa is expressed in higher levels than OPNb and OPNc variants (Figure 2).

OPNa overexpression induce the formation of calcium deposits in c643 thyroid cells

The ability of c643 thyroid cells overexpressing each OPN-SV to produce calcified extracellular matrix (ECM) was evaluated by Alizarin Red staining following 31 days in culture (Figure 3). We found that c643 cells overexpressing OPNa variant presented higher Alizarin Red staining intensity, when compared to those overexpressing OPNb and OPNc variants. These results showed that OPNa-overexpressing cells were able to produce higher rates of calcified ECM (Figure 3, left panel).

OPNa promotes collagen synthesis in thyroid cell line

Since PB formation is described to rely on calcium deposition, in association with some ECM component, such as collagen [23], we decided to analyse if the OPN-SV differently contribute to induce collagen deposition in c643 cells. For this, c643 OPN-SV overexpressing cells were maintained in culture for 31 days and stained with Masson trichrome. We observed that OPNa overexpression prompted substantial collagen synthesis, when compared with the other variants (OPNb and OPNc), as shown by the Masson trichrome dark purple-red staining (Figure 3, right panel).

TPC1 thyroid cell line produce calcium deposits and collagen synthesis

Since our previous study had shown that from the nine thyroid cancer cell lines for OPN expression [19], TPC1 was the one presenting the highest endogenous levels of OPNa variant we decided to analyse if this cell line was also able to deposit calcium and to promote collagen synthesis, without exogenous OPN-SV overexpression. Our results

showed that TPC1 cells are also able to promote calcification and collagen synthesis in the ECM forming PB-like structures (Figure 3, upper).

4.4 Discussion

In this study, we investigated the association between the expression of the matricellular protein OPN and its splice variants in cPTC cases and their correlations with the presence of PB in such cases. We observed that cPTC tumour cases presenting PB are associated with younger age and the presence of lymph node metastasis. We also found that higher OPNa transcript expression is correlated with the presence of PB in cPTC cases. Noteworthy, OPNa variant overexpression strongly induced calcified collagen-rich matrix deposition, forming PB-like structures in thyroid cancer cell lines *in vitro*.

We have shown that tOPN protein expression has been observed in cPTC samples that present PB. Of note, in the immunohistochemistry analysis of the cases stained for tOPN, PB are much clearer than in HE staining, particularly since the staining of PB with anti-OPN antibody is stronger. To date, only two studies reported an association between OPN expression and the occurrence of PB in PTC samples [17, 18]. Both studies showed that the expression of tOPN protein associates with the occurrence of PB in PTC samples, suggesting a role for OPN in calcification of PTC [17]. Another association between OPN and calcification in cancer was reported by Hirota *et al*. [24], who described OPN protein co-localization with calcium phosphate in meningioma tissues, further evidencing a role for OPN on the PB formation in this context.

We further showed that cPTC cases that presented PB also had higher expression levels of tOPN, OPNa, OPNb and OPNc transcripts, although only OPNa and OPNb levels demonstrated statistically significant differences (OPNa > OPNb). In a previous work, we demonstrated that among the three OPN-SV, OPNa had the highest expression

levels in cPTC, when compared with other thyroid tissues [19]. Therefore, the current results are in accordance with our earlier observations, raising a possible relation between OPNa variant overexpression and the formation of PB in cPTC. Regarding the small series of diffuse sclerosing PTC samples analysed in this study, no expression of tOPN protein was observed. On the other hand, when we evaluated the three OPN-SV in these cases, high OPNa levels have been detected. In addition to the cPTC, the diffuse sclerosing PTC is the variant that frequently presents numerous PB [25], as also confirmed in the 3 cases analysed here. Notably, diffuse sclerosing PTC have been described to have a higher incidence of lymph node metastasis when compared to cPTCs [26].

Regarding the analysis of cPTC clinicopathological features, we found a correlation between cPTC cases presenting PB and presence of lymph node metastasis and younger patients. PB is a diagnostic indicator for cPTC, and its presence strongly suggest tumour malignancy in preoperative diagnosis. Bai *et al.* [12] also reported that the presence of PB in PTC cases was associated with gross lymph node metastasis and high-stage cancer (stage IVa). Conversely, Pyo *et al*. [27] found an association between PB and tumour multifocality, extrathyroid extension, and lymph node metastasis. The higher expression of OPNa in cPTC containing PB and the association of PB with lymph node metastases is in accordance with our previous data, in which we showed that OPNa overexpression contributes to cPTC progression features [19]. Bai *et al*. [12] found an association between stromal calcification and advanced age of patients (> 60 years). However, in that study authors only considered calcification in the stroma. Stromal calcification frequently arises in benign lesions, whereas PB are suggestive of malignancy. Herein, we evaluated only PB in cPTC cases, excluding other dystrophic or

stromal calcifications, as well as bone formation. In fact, when we consider dystrophic calcification, no correlation was found with OPNa expression (*data not shown*).

In our previous work, we also reported an association between tOPN protein expression and presence of stromal tissue areas in cPTC cases, suggesting that this may be correlated with tumour aggressiveness in cPTCs [19]. Herein, we further investigated this point and presented *in vitro* and *in vivo* evidence of an association particularly between OPNa expression and the presence of PB and stromal collagen.

In order to establish putative impacts of OPN-SV regarding the calcification process, OPNa, OPNb and OPNc (and an empty vector as a control) were overexpressed in c643 TC cells. We then analysed the effects of OPN-SV overexpression in the formation of calcium deposits in the ECM produced by the cells and found that OPNa overexpressing cells strongly induced calcium deposits in the ECM. Calcification within the thyroid gland is a common finding both on thyroid imaging and thyroid histopathologic findings [28]. Although PB are a highly specific sign of malignancy frequently detected in cPTC [27], the mechanisms by which PB are formed in thyroid tumours is still controversial. Several reports have revealed that calcification is more common in malignant than in benign thyroid nodules [29, 30]. Other authors reported that intrathyroidal calcification was noted in 26.1% (29 out of 111) of the malignant thyroid nodules and in only 8.0% (20 out of 250) of benign thyroid samples [28]. Nonetheless, it is important to mention that many authors have been stressing that the presence of intrathyroidal calcification *per se* cannot be used to distinguish between benign and malignant thyroid disease [29-32].

We also observed that, among the overexpressing OPN-SV c643 cells, OPNa overexpressing cells induced more efficiently collagen synthesis in the ECM. This data indicates that among the three variants, OPNa remarkably promoted these effects. These

results are in accordance with our previous observations, whereby OPNa (among all OPN-SV) seemed to be the key OPN variant associated with the presence of stroma [19].

The roles of OPN in the tumour ECM microenvironment has been investigated. For instance, Liaw *et al*. [33] showed that OPN knockout mice exhibit defective repair of incisional skin wounds with abnormal collagen fibrillogenesis. Additionally, it is known that depending on the ECM stiffness, collagen fibbers potentiate cell migration, especially in the presence of MMPs activity [34]. Accordingly, our previous work demonstrated that OPNa activates MMP2 and MMP9 activities in thyroid cell lines, besides promoting cell migration and invasion in the *in vivo* CAM model [19]. Being the most abundant ECM components, collagens constitute up to 90% of the ECM and 30% of the total proteins in humans, also providing the structural integrity and the tensile strength of human tissues and organs [35]. Furthermore, the increased collagen deposition is the most well-recognized ECM alteration in tumour tissue [36-38]. In the context of cancer biology, it has also been shown that collagens regulate the physical and the biochemical properties of tumour microenvironment, modulating cancer cell polarity, migration and signalling [39-41].

In order to evaluate OPNa properties on inducing calcium deposit and collagen synthesis in the ECM, we selected TPC1 among nine thyroid cell lines, due to its highest levels of endogen OPNa expression. Similarly to what has been observed for c643 cells overexpressing OPNa variant, TPC1 cells also exhibited calcium deposits and production of collagen fibbers. TPC1 cell line is recognized as presenting the *RET/PTC* rearrangements [42]. This molecular alteration was demonstrated to induce OPN expression in thyroid cell lines, such as PCCl3 [43], reinforcing the hypothesis that OPN plays a role in such processes. Although our results also provide evidence in the same direction, in particular giving some early evidence that OPNa plays a role in such

mechanism, further studies are needed to better understand how OPNa modulates collagen synthesis and the calcification process. In addition, it is imperative to better comprehend the underlying molecular mechanisms that dictate the lymphovascular invasion, thus contributing to the pathophysiology knowledge of cPTCs.

In conclusion, our results show that the presence of PB is associated with lymph node metastases in cPTC samples. Additionally, OPNa overexpression and its association with the occurrence of PB in cPTC samples, together with its strong capacity to promote calcium deposit and to synthesize collagen in the ECM in thyroid cells *in vitro*, supports an important role for OPNa in the etiopathogenesis of the cPTC.

Disclosure of Potential Conflicts of Interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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4.6 Figures/Figure Legends

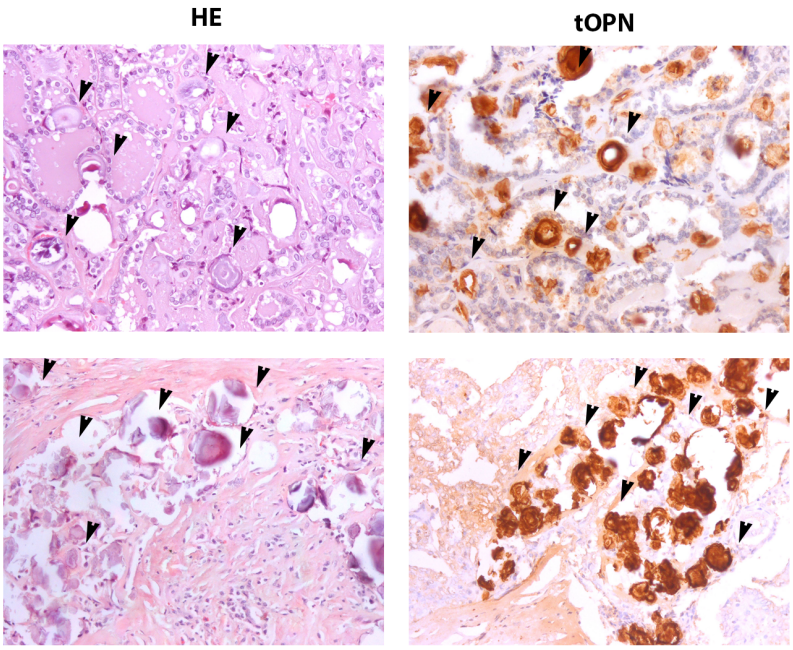

Figure 1: Total OPN staining at PB in cPTC cases. Two different representative cPTC cases (upper and lower panel) showing psammoma bodies (PB) appearing rounded, sometimes fused with each other, or even fragmented, with concentric lamination, as shown by the black arrow heads. Left panel: PB stained by HE, 40 x. Right panel: PB stained for tOPN antibody, 40 x.

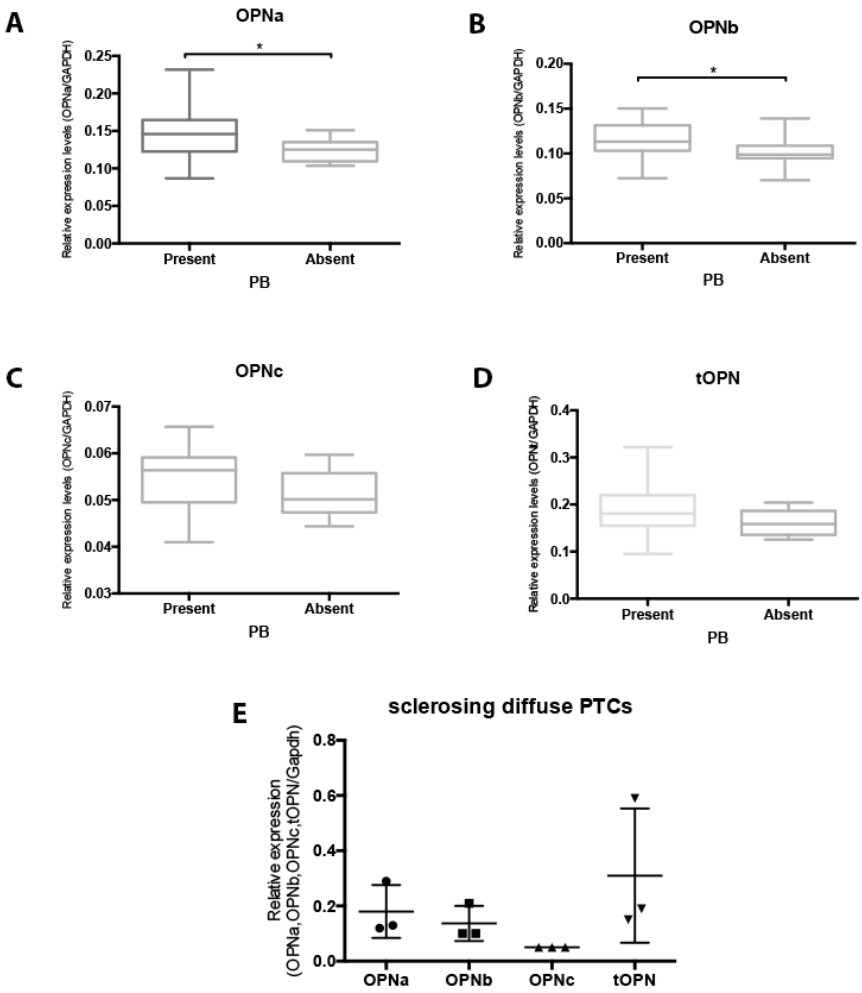

Figure 2: Expression levels of tOPN, OPNa, OPNb and OPNc transcripts in cPTC samples concerning presence or absence of PB. (A) tOPN, (B) OPNa, (C) OPNb and (D) OPNc mRNA expression levels measured by real time PCR in the cPTC samples. (E) OPNa, OPNb, OPNc and tOPN mRNA expression levels measured by real time PCR in the three diffuse sclerosing PTC samples. * $p < 0.05$. Results are from at least two independent assays with triplicates.

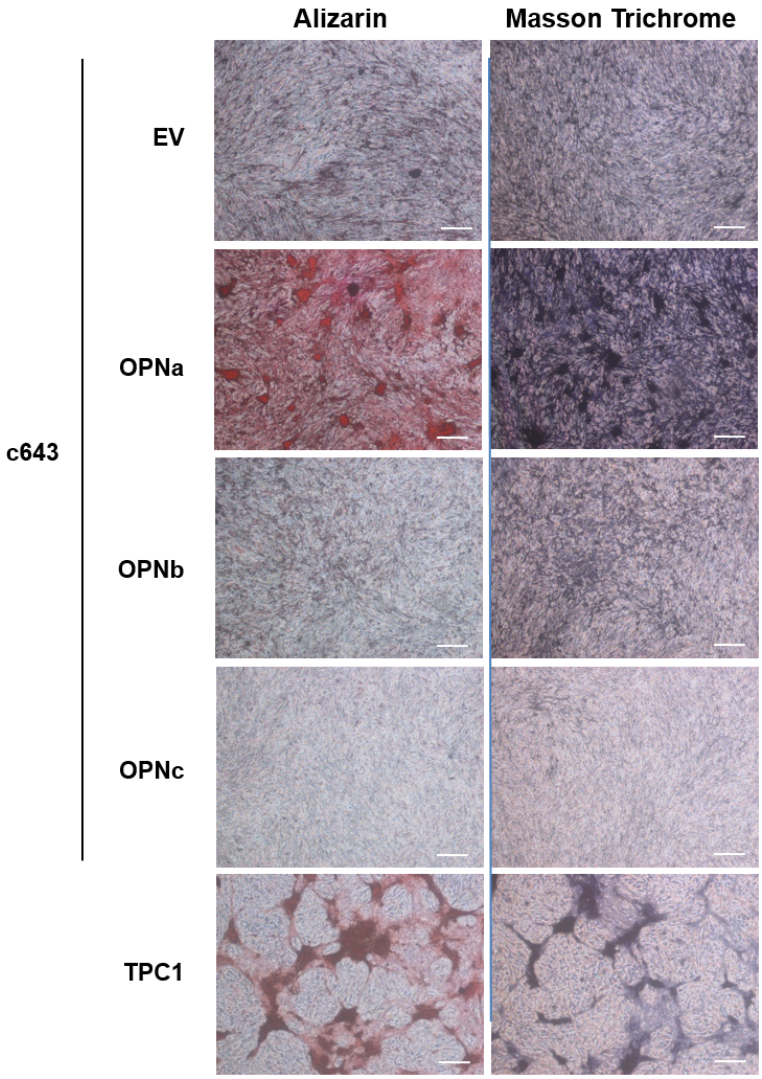

Figure 3: c643 cells overexpressing OPNa and TPC1 cells present higher calcification and collagen ECM production levels. Left panel: Matrix calcification detected with Alizarin Red staining. Dark orange areas correspond to extracellular matrix rich in calcium deposits. Representative photomicrographs of 2 independent experiments are shown. Right panel: Collagen ECM production was determined by Masson trichrome staining. Dark purple areas correspond to extracellular matrix rich in collagen. Scale bar: 100 µM. Representative photomicrographs of 2 independent experiments at 24 days of culture are shown.

Tables:

Table 1: Summary of the clinical, pathological and molecular data of the cPTC cases

Variable	cPTC n (%)
Sex*	
Male	6 (15)
Female	41 (85)
Age (yr) (mean ±S.D.)*	39.87 (±16.42)
<45	26 (56.5)
≥45	20 (43.4)
Stroma	
Absent	21 (44)
Present	27 (56)
Tumour size (cm)*	
(mean ±S.D.)	2.4 (±1.1)
<2	17 (37)
≥2	29 (63)
Capsule*	
Absent	24 (56)
Present	19 (44)
Psammoma Bodies*	
Absent	21 (47)
Present	24 (53)
Extrathyroid Extension*	
Absent	24 (57)
Present	18 (43)
Invasion Lympho (vascular and/or capsular)*	
Absent	15 (35)
Present	28 (65)
Lymph Node Metastases*	
Absent	21 (60)
Present	14 (40)
RET/PTC1* translocation	
Absent	37 (84)
Present	7 (16)
BRAF*^{V600E} mutation	
Absent	22 (48)
Present	24 (52)
RAS* mutation	
Absent	44 (96)
Present	2 (4)

*Clinicopathological data from all the cases included in the series was impossible to obtain

Table 2: Staining score of tOPN IHC in cPTC samples.

OPN Staining Score*	n	%
0	19	39.6
1	1	2.1
2	7	14.6
3	6	12.5
4	2	4.2
5	7	14.6
6	5	10.4
7	1	2.1

* Staining intensity plus % of positive stained cells

Table 3. tOPN protein expression evaluated by IHC and correlation with presence of PB in formalin-fixed paraffin-embedded (FFPE) cPTC samples

Psammoma bodies (PB)	N %	Score Mean	
No	21 (47)	2.29	
Yes	24 (53)	2.54	p=0.71
Total	45 (100)		

Table 4: Correlation between tOPN protein expression and the clinicopathological characteristics in cPTC

Variable	N (%)	OPN Score Mean	p value
Sex*			
Male	6 (13)	1.50	0.34
Female	41 (87)	2.49	
Age (years)*			
<45	26 (55)	2.15	0.48
≥45	20 (45)	2.65	
Stroma			
Absent	21 (43)	1.48	**0.01**
Present	27 (57)	3.04	
Tumour Size (cm)*			
< 2	17 (37)	2.94	0.22
≥2	29 (63)	2.07	
Extrathyroid Extension*			
Absent	24 (57)	1.79	0.09
Present	18 (43)	3.00	
Invasion Lympho (capsular and/or vascular)*			
Absent	15 (35)	1.93	0.49
Present	28 (65)	2.46	
Lymph Node Metastasis*			
	21 (60)	2.33	0.71
Absent	14 (40)	2.64	
Present			
RET/PTC* rearrangement			
Absent	37 (84)	2.57	0.88
Present	7 (16)	2.43	
BRAF*^{V600E} mutation			
Absent	22 (48)	2.64	0.62
Present	24 (52)	2.29	
RAS* mutation			
Absent	44 (96)	2.57	0.11
Present	2 (4)	0	

*Clinicopathological data from all the cases included in the series was impossible to obtain

Table 5: Correlation between the presence of PB and the clinicopathological characteristics in cPTC

	Psammoma Bodies (PB)		
Total n = 45	Absent n = 21 (%)	Present n = 24 (%)	p value
Sex			
Male	2 (9.5)	2 (8.3)	NS (1.0)
Female	19 (90.5)	22 (91.7)	
Age (years)			
<45	7 (33.3)	18 (75)	**0.005**
≥45	14 (66.7)	6 (25)	
Stroma			
Absent	11 (52.4)	8 (33.3)	NS (0.1)
Present	10 (47.6)	16 (66.7)	
Tumour size (cm)			
<2	10 (47.6)	8 (34.8)	NS (0.3)
≥2	11 (52.4)	15 (65.2)	
Capsule			
Absent	9 (47.4)	14 (63.6)	NS (0.2)
Present	10 (52.6)	8 (36.4)	
Extrathyroid Extension			
Absent	10 (58.8)	13 (56.5)	NS (0.8)
Present	7 (41.2)	10 (43.5)	
Invasion Lympho (Vascular and/or capsular)			
Absent	9 (47.4)	4 (19)	NS (0.1)
Present	10 (52.6)	17 (81)	
Lymph node metastases			
Absent	11 (84.6)	9 (42.9)	**0.030**
Present	2 (15.4)	12 (57.1)	
***RET/PTC* translocation**			
Absent	18 (90)	17 (77.3)	NS (0.4)
Present	2 (10)	5 (22.7)	
***BRAF*^{V600E} mutation**			
Absent	9 (42.9)	12 (52.2)	NS (0.5)
Present	12 (57.1)	11 (47.8)	
***RAS* mutation**			
Absent	20 (95.2)	22 (95.7)	NS (1.0)
Present	1 (4.8)	1 (4.3)	

Abbreviations: NS, not significant. Numbers in parentheses represent percentages within each category. Bold values indicate the result was statistically significant.

Table 6: Predictive factors for lymph node metastases in papillary thyroid carcinomas

		Lymph Node Metastases (n = 34)			
	Presence	**Univariate Analysis**		**Multivariate Analysis**	
	(%)	**OR (95% CI)**	***P* value**	**OR (95% CI)**	***P* value**
Total	14 (41)				
Sex					
Male	1 (7.1)	1 (Referent)			
Female	13 (92.9)	1.4 (0.8 – 25)	NS (0.8)		
Age, years					
< 45	11 (78.6)	4.4 (0.9 – 21)	**0.05**	3.1 (0.5 – 16)	NS (0.1)
≥ 45	3 (21.4)	1			
Stroma					
Absent	4 (28.6)	1			
Present	10 (71.4)	1.5 (0.3 – 6.5)	NS (0.5)		
Tumour size (cm)					
< 2	5 (35.7)	1			
≥ 2	9 (64.3)	1.3 (0.3 – 5.4)	NS (0.6)		
PB					
Absent	2 (14.3)	1			
Present	12 (85.7)	7.3 (1.2 – 41)	**0.02**	5.6 (0.9 – 34)	**0.05**
Capsule					
Absent	8 (61.5)	2.1 (0.5 – 8.7)	0.2		
Present	5 (38.5)	1			
Extrathyroid extrension					
Absent	6 (46.2)	1			
Present	7 (53.8)	2.0 (0.4 – 8.3)	NS (0.3)		
Invasion Lympho (Vascular and/or Capsular)					
Absent	2 (14.3)	1			
Present	12 (85.7)	4.3 (0.7 – 25)	NS (0.09)		
***BRAF*^{V600E} mutation**					
Absent	8 (61.5)	1			
Present	5 (38.5)	2.1 (0.5 – 8.7)	NS (0.2)		

Table 7: Correlation between tOPN and OPN-SV transcript expression levels with the presence of PB in cPTC samples

Variable	tOPN mRNA expression (Median)	p-value	OPNa mRNA expression (Median)	p-value	OPNb mRNA expression (Median)	p-value	OPNc mRNA expression (Median)	p-value
Psammoma Bodies								
Absent (n=19)	0.15	p=0.73	0.12	**p=0.01**	0.10	**p=0.04**	0.052	p=0.07
Present (n=26)	0.18		0.14		0.11		0.055	

Chapter 5 – Paper III - Osteopontin expression is correlated with differentiation and good prognosis in medullary thyroid carcinoma

This chapter appears as an article with the same title published in the “European Journal of Endocrinology” Ferreira LB et al. (2016) 174, 551–561

Osteopontin expression is correlated with differentiation and good prognosis in medullary thyroid carcinoma

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Abstract

Background: Osteopontin (OPN) or secreted phosphoprotein 1 (SPP1) is a matricellular glycoprotein whose expression is elevated in various types of cancer and has been shown to be involved in tumourigenesis and metastasis in many malignancies, including follicular cell-derived thyroid carcinomas. Its role in C-cell-derived thyroid lesions and tumours remains to be established.

Objective: The objective of this study is to clarify the role of OPN expression in the development of medullary thyroid carcinoma (MTC).

Methods: OPN expression was analysed in a series of 116 MTCs by immunohistochemistry and by qPCR mRNA quantification of the 3 OPN isoforms (OPNa, OPNb and OPNc) in six cases from which fresh frozen tissue was available. Statistical tests were used to evaluate the relationship of OPN expression and the clinicopathological and molecular characteristics of patients and tumours.

Results: OPN expression was detected in 91 of 116 (78.4%) of the MTC. We also observed high OPN expression in C-cell hyperplasia as well as in C-cells scattered in the thyroid parenchyma adjacent to the tumours. OPN expression was significantly associated with smaller tumour size, *PTEN* nuclear expression and *RAS* status, and suggestively associated with non-invasive tumours. OPNa isoform was expressed significantly at higher levels in tumours than in non-tumour samples. OPNb and OPNc presented similar levels of expression in all samples. Furthermore, OPNa isoform overexpression was significantly associated with reduced growth and viability in the MTC-derived cell line (TT).

Conclusion: The expression of OPN in normal C-cells and C-cell hyperplasia suggests that OPN is a differentiation marker of C-cells, rather than a marker of biological aggressiveness in this setting. At variance with other cancers, OPN expression is associated with good prognostic features in MTC.

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Introduction

Thyroid tumours are the most common malignancies of the endocrine system. About 1% of the thyroid cells correspond to (parafollicular) C-cells that are located in the basal layer of thyroid follicles (1). Medullary thyroid carcinoma (MTC) is the thyroid tumour that arises from the neural crest-derived C-cells of the thyroid gland. MTCs account for 5–10% of the clinically evident thyroid cancers and are associated to a higher incidence of distant metastases and poorer prognosis, when compared with follicular cell-derived well-differentiated thyroid carcinomas (2, 3). In total, 70–80% of MTCs are sporadic and the remaining 20–30% are hereditary (1).

C-cells are dispersed as individual cells or in small groups in the spaces among thyroid follicles, and their function is to secret a whole series of regulatory peptides and growth factors (4). Hyperplasia of C-cells corresponds to an abnormal increase in the number of these cells and is recognised as a precursor of medullary carcinoma (especially in the setting of multiple endocrine neoplasia types IIA and IIB) and associates with a number of other conditions, including advanced age, hypercalcemia, hypergastrinemia due to Zollinger–Ellison syndrome, Hashimoto disease and chronic lymphocytic thyroiditis, follicular and papillary thyroid neoplasms, exogenous estrogen administration and cimetidine treatment. Yet, patients with germ-line mutations of the *RET* oncogene present age-related progression of C-cell lesions from C-cell hyperplasia (CCH) towards MTC (5). In sporadic cases, the putative role of CCH in carcinogenesis is controversial.

Thyroid carcinomas frequently present aberrant expression of different gene products (6, 7, 8), and it was advanced that such overexpression can be related to cancer progression. Castellone *et al.* (9) demonstrated that OPN is one of the most overexpressed molecules in papillary thyroid carcinoma (PTC) and showed that its up-regulation promoted proliferation, migration and spreading of thyroid cancer-derived cell lines (9).

OPN is a secreted multifunctional phosphorylated glycoprotein constitutively expressed in epithelial cells of the gastrointestinal, reproductive and urinary tracts, sweat ducts, lung bronchi, pancreas, lactating breast, salivary glands and, notably, in the gallbladder (10). OPN mRNA and/or protein expression levels were reported to be increased in several types of human cancers, such as prostate carcinoma (11); melanoma (12); and ovary (13), lung and gastric carcinoma (14). OPN overexpression was associated with poor prognosis in some malignancies, namely, in PTC (15). The overexpression of OPN in many types of tumours and its frequent association with invasive properties and aggressive clinicopathological features (16, 17) suggest that OPN may be involved in tumourigenesis, tumour progression and in metastasis formation (18, 19). In contrast to this, OPN expression has been related with better prognostic clinical features in osteosarcoma (20). To date, it has not been described the expression of OPN in neuroendocrine tumours if one excludes a case report of Kuroda *et al.* (21) showing the expression of OPN in neoplastic cells of a large-cell neuroendocrine carcinoma (LCNEC) of the lung. Kuroda *et al.* (21) observed that part of the LCNEC neoplastic cells as well as the calcified foci were positive for OPN, suggesting that in addition to dystrophic calcification, OPN may be involved in the mechanism of calcification within necrotic areas of LCNEC.

OPN mRNA is subjected to alternative splicing that generates three protein variant isoforms, designated as OPNa, OPNb and OPNc (22). The three OPN isoforms have been shown to play specific roles in different types of tumours; for example, in breast and ovarian cancer cells, OPNc isoform activates invasion and adhesion properties (13, 23). In prostate cancer cells, OPNb and OPNc stimulate proliferation, migration and invasion (11), and in hepatocellular carcinoma cells, OPNa and OPNb promote migration (24).

To the best of our knowledge, expression of total OPN and of OPN splicing variants has not been systematically assessed so far in MTC samples. The objective of this study was therefore to evaluate the expression profile of OPN in MTC and to relate such expression with molecular and clinicopathological data.

Subjects and methods

Human MTC tissue samples

The study involved 116 cases of MTC (diagnosed in three Institutes from 1974 to 2011). Formalin-fixed, paraffin-embedded tissue and clinical data were retrieved from the files of Centro Hospitalar *São* João (CHSJ)/Medical Faculty of Porto (FMUP)/Ipatimup (56 cases), Portuguese Institute of Oncology, Coimbra (IPO-C) (19 cases), and Portuguese Institute of Oncology, Lisboa (IPO-L) (41 cases). The diagnosis of MTC was revised by two pathologists (CE and MSS) and confirmed by calcitonin immunostaining. Clinicopathological and follow-up data were obtained from the surgical pathology reports and patients' records

of the Department of Pathology and Oncology of CHSJ and from IPO databases (Table 1). *RET* and *RAS* genetic characterisation of the series had been done previously and reported in two publications (25, 26). The study was approved by the Hospital Ethical Committees, and National Ethical rules were followed in every procedure.

Immunohistochemistry

Semi-quantitative immunohistochemistry (IHC) analysis of OPN expression was performed in representative 116 MTC tumour tissue sections using an antibody that recognises all OPN isoforms (anti-total OPN). To demonstrate specificity of the antibody to human OPN, we used normal gallbladder as positive control, because it has been previously reported to strongly express OPN in both supranuclear and luminal contents (10) (Fig. 1A). The following was the IHC procedure: briefly, deparaffinised and rehydrated sections were subjected to microwave treatment in 10 mM sodium citrate buffer, pH 6.0, for antigen retrieval. After blocking, the sections were incubated overnight at 4 °C in a humidified chamber with a primary antibody anti-OPN (polyclonal, goat, 1:500; R&D Systems,

Table 1 Summary of the clinical, pathological and molecular data of the MTC cases.

	MTC
Gender ($n=97$)*	
Female	53 (54.6%)
Male	44 (45.4%)
Tumour size (cm)* ($n=80$; mean±S.D.)	3.00 (±2.24)
Stroma ($n=112$)*	
Absent	31 (27.7%)
Present (hyaline)	81 (72.3%)
Amyloid deposits ($n=42$)*	
Absent	16 (38.1%)
Present	26 (61.9%)
Extrathyroidal extension (n=64)*	
Absent	28 (43.8%)
Present	36 (56.3%)
Metastasis (lymph node and/or distance) ($n=73$)*	
Absent	27 (36.9%)
Present	46 (63.1%)
Invasion (vascular and/or capsular) ($n=34$)*	
Absent	6 (17.6%)
Present	28 (82.4%)
RET mutation ($n=116$)	
Absent	49 (42.2%)
Present	67 (57.8%)
RAS mutation ($n=116$)	
Absent	97 (83.6%)
Present	19 (16.4%)
PTEN nuclear score ($n=75$)*	
Low	48 (64.0%)
High	27 (36.0%)
PTEN cytoplasmic score ($n=75$)*	
Low	14 (18.7%)
High	61 (81.3%)
pS6 intensity score ($n=75$)*	
Low	33 (44.0%)
High	42 (56.0%)

*Clinicopathological data from all the cases included in the series was impossible to obtain.

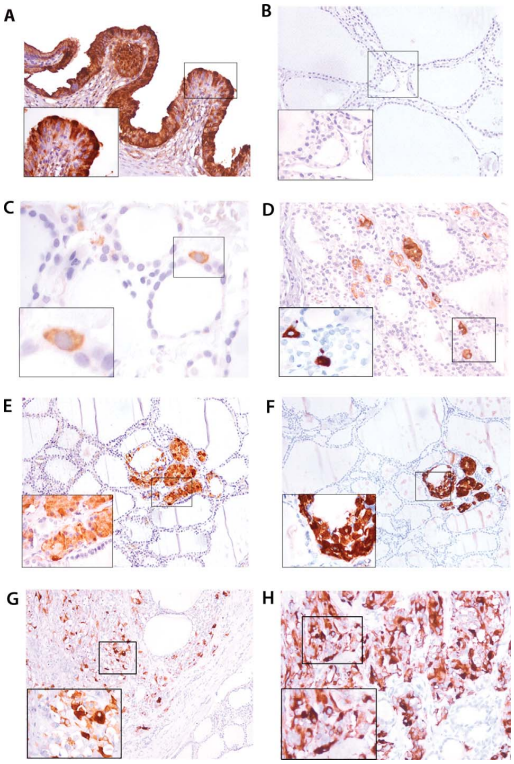

Figure 1
OPN cytoplasmic intracellular immunoexpression. (A) Staining in normal gallbladder used as positive control. (B) Normal thyroid gland showing no OPN staining. (C) Normal thyroid tissue showing OPN staining in scattered C-cells. (D) 'Normal' thyroid tissue adjacent to MTC; note the OPN positive scattered C-cells (in the inset calcitonin staining of the same area). (E) C-cell hyperplasia showing OPN staining (F) The same area of C-cell hyperplasia stained for calcitonin. (G) A case of MTC showing OPN staining in many neoplastic cells, classified as score: 6 (proportion of positive cells: 50–75%+ staining intensity: 3+). (H) The same case stained for calcitonin showing positivity of almost every neoplastic cells. A full colour version of this figure is available at http://dx.doi.org/10.1530/EJE-15-0577.

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Minneapolis, MN, USA). The detection was performed with a labelled streptavidin–biotin immunoperoxidase detection system (Thermo Scientific/ Lab Vision, Fremont, USA), and the IHC staining was developed with 3,3′-diaminobenzidine substrate. Negative control consisted in the omission of the primary antibody incubation. Semi-quantitative OPN expression was evaluated using a staining score, which has been independently established by two observers (CE and LF). Proportion of positive-stained cells was scored as <5%=0, 5–25%=1, 25–50%=2, 50–75%=3 and >75%=4, while staining intensity values were classified as absent=0, faint=1, moderate=2 or strong=3 (Table 2). The OPN staining score (range from 0 to 7) corresponds to the sum of staining intensity and the proportion of positive-stained cells (Table 3). OPN staining score was correlated with data previously obtained by our group in this tumour series with regards to *PTEN* and pS6 IHC expression (25).

Cell culture and generation of medullary cancer cells overexpressing OPN isoforms

As a model to examine the putative roles of OPN isoforms in MTC, we used a MTC-derived cell line (TT). TT cell line was cultured in RPMI 1640 supplemented with 20% of foetal bovine serum, 100 IU/ml penicillin and 100 mg/ml streptomycin in a humidified environment containing 5% CO_2 at 37 °C. The open reading frame of OPN splicing variants, OPNa, OPNb and OPNc, were cloned into pCR3.1 mammalian expression vector as previously described (23), and the DNA constructs were used for transfection into TT cells. Transfections were carried out using Lipofectamine 2000 (Invitrogen). The OPN isoforms/pCR3.1 plasmids or the vector alone (empty vector (EV)) were transfected into TT cells, and the stably expressing cells were selected with 600 μg/ml of G418 in the culture medium. The cells overexpressing each OPN isoform were tested individually on functional assays, as indicated below.

Table 2 Staining intensity and proportion of positive stained cells of OPN IHC in 116 MTC cases.

	Frequency (*n*)	Percentage (%)
Staining intensity		
Absent	25	21.6
Faint	31	26.7
Moderate	25	21.6
Strong	35	30.1
Total	116	100
Proportion of positive stained cells		
<5%	31	26.7
5–25%	12	10.3
25–50%	19	16.4
50–75%	12	10.3
75–100%	42	36.2
Total	116	100

Table 3 Staining score of OPN IHC in 116 MTC cases.

OPN staining score*	Frequency (*n*)	Percentage (%)
0	25	21.6
1	1	0.9
2	3	2.6
3	14	12.1
4	16	13.8
5	27	23.3
6	18	15.5
7	12	10.3
Total	116	100%

*Staining intensity+proportion of positive-stained cells.

RNA extraction and reverse transcription

Total RNA was extracted from frozen specimens of MTC ($n=6$), adjacent normal tissue specimens ($n=6$), two MTC-derived cell lines (TT and MZ-CRC-1) and from TT cells overexpressing OPNa, OPNb, OPNc and EV using a Trizol commercial kit (Life Technologies, GIBCO BRL), according to the manufacturer's protocol. RNA was quantified by spectrophotometry, and its quality was checked by analysis of 260/280 and 260/230 nm ratios. For cDNA preparation, 1 μg of total RNA was reverse transcribed using the RevertAid first-strand cDNA synthesis kit (Fermentas, Burlington, ON, Canada).

Real-time PCR

The amplification of fragments corresponding to each OPN isoform was performed using the following oligonucleotide pairs: OPNa: 5′-ATC TCC TAG CCC CAC AGA AT-3′ (forward) and 5′-TTC TCC ATG GTG GTG AAG ACG CCA-3′ (reverse); OPNb: 5′-CTC CTA GCC CCA CAG ACC CT-3′ (forward) and 5′-TAT CAC CTC GGC CAT CAT ATG-3′ (reverse); OPNc: 5′-CTG AGG AAA AGC AGA ATG-3′ (forward) and 5′-AAT GGA GTC CTG GCT GT-3′ (reverse). GAPDH was amplified with primers 5′-TCC CAT CAC CAT CTT CCA GGA GCG-3′(forward) and 5′- TGG CGT CTT CAC CAC CAT GGA GAA-3′(reverse) and was used as an internal control to normalise the expression and to verify integrity of the cDNA. All qRT-PCRs were conducted using SYBR Green (Applied Biosystems). Gene expression of the target gene was calculated by using the ΔCT method.

Immunocytochemistry

Upon being plated on coverslips, cells were fixed in 4% paraformaldehyde for 20 min at room temperature (RT). Cells were emerged in NH_4Cl 50 mM in PBS during 10 min, and then, cells were permeabilised in 0.2% Triton X-100 and blocked in 5% BSA in PBS for 30 min at RT. Primary antibodies were diluted in PBS containing 5% BSA and incubated overnight at 4 °C as follows: rabbit polyclonal against OPN (Rockland, Limerick, PA, USA, diluted 1:500). Coverslips were washed in 0.1% Triton X-100 prepared in PBS (PBT) and incubated with goat anti-rabbit IgG secondary antibodies conjugated with Alexa Fluor 594 (Invitrogen; diluted 1:300 in 5% BSA–PBT) for 1 h at RT. Nuclei were stained with 0.1 mg/ml diamino phenylindole (DAPI; Sigma–Aldrich). Images were taken by a Zeiss fluorescence microscope with ApoTome attachment (Axio Imager Z1 stand).

Cell growth and viability assays

Cell growth was analysed by MTT assay. Cells over-expressing OPNa, OPNb, OPNc and EV were seeded in 96-well plates at 5×10^4 cells density per well. After 48 h for adherence of the cells, 5 mg/ml of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) were added to each well. Cells stayed in the incubator at 37 °C, 5% CO_2 for 4 h, and then dimethyl sulphoxide was added in the cells for solubilisation of MTT. Absorbance was measured at 570 nm. Viability assay was performed by Trypan blue exclusion after 48 h of plating the cells. Three independent experiments were carried out using triplicates in each experiment. Data are expressed as mean ± S.E.M. and analysed using Mann–Whitney test. Significance level was fixed in 0.05.

Statistical analysis

Statistical analysis was performed using 22.0 SPSS statistical package (IBM, 2014). χ^2 and independent samples *t*-test were performed to verify if there was any association(s) between OPN expression and clinicopathological data. For *in vitro* experiments, data are expressed as mean ± S.E.M. and analysed using Mann–Whitney test. Values of $P\leq0.05$ were considered statistically significant.

Results

OPN immunoexpression in MTC, C-cell hyperplasia and C-cells

IHC studies were performed in samples of the 116 MTC cases. Stromal areas of MTC, when present, were virtually negative for OPN staining (Fig. 1B). OPN protein expression was found in 91 of 116 MTCs (78.4%). In the positive cases, the staining intensity was faint in 26.7%, moderate in 21.6% and strong in 30.1% of the cases (Table 2); on average, more than 50% of the cells were positive (Table 2). Among the positive cases, staining score was 1 in 0.9%, 2 in 2.6%, 3 in 12.1%, 4 in 13.8%, 5 in 23.3%, 6 in 15.5% and 7 in 10.3% of the cases (Table 3).

OPN staining was mainly localised in the cytoplasm, i.e. intracellular. Representative regions of OPN and calcitonin staining are depicted in Fig. 1G and H. We also observed strong OPN staining in dispersed C-cells (Fig. 1C and 1D), as well as in C-cell hyperplasia foci detected in the adjacent thyroid tissue of some samples (Fig. 1E), which was confirmed by calcitonin positive staining (Fig. 1F).

Association of OPN immunoexpression with clinicopathological and molecular features of MTC

We observed significant differences of OPN staining score with regards to tumour size, *PTEN* nuclear expression and *RAS* status. Tumours larger than 2 cm presented a lower average OPN staining score than tumours smaller than 2 cm (2.8 vs 4.6 respectively; $P=0.001$). Tumours displaying *PTEN* nuclear expression showed a higher average of OPN staining score than tumours without *PTEN* nuclear expression (5.3 vs 3.9 respectively; $P=0.003$). Finally, tumours WT for *RAS* presented a higher average staining score for OPN than tumours harbouring a *RAS* mutation (4.0 vs 2.6 respectively; $P=0.043$). There was a suggestively significant association with invasion (capsular and/or vascular): tumours without evidence of invasion presented a higher OPN staining score than tumours presenting invasion (4.8 vs 3.5 respectively; $P=0.083$). No significant or suggestive associations were observed between OPN staining score and gender or age of the patients, presence of stroma, lymph node and/or distant metastasis, presence of amyloid, extrathyroidal extension, *RET* mutation, *PTEN* cytoplasmic expression and pS6 expression (Table 4).

Expression of OPN isoforms in MTC

In order to further investigate the OPN expression profile in MTC samples, we advanced that determining OPN splicing isoform expression could provide evidence regarding the contribution of each OPN variant within the total OPN expression evaluation. To achieve this objective, we evaluated mRNA levels of OPNa, OPNb and

Table 4 Clinicopathological and molecular associations with OPN expression. Data is presented as mean±S.D.

Clinicopathological	*n*	OPN immunoexpression	*P* value
Gender			0.196
Female	53	(3.9±2.3)	
Male	44	(3.3±2.4)	
Tumour size (cm)			**0.001**
<2	30	(4.6±1.8)	
≥2	50	(2.8±2.4)	
Stroma			0.492
Absent	31	(3.6±2.4)	
Present (hyaline)	81	(4.0±2.2)	
Amyloid deposits			0.239
Absent	16	(4.8±2.0)	
Present	26	(4.0±2.2)	
Extrathyroidal extension			0.620
Absent	28	(3.4±2.2)	
Present	36	(3.1±2.5)	
Metastasis			0.434
Absent	27	(3.0±2.3)	
Present	46	(3.4±2.5)	
Invasion (vascular and/or capsular)			**0.083**
Absent	6	(3.5±2.4)	
Present	28	(4.8±1.3)	
RET			0.883
Normal	49	(3.7±2.3)	
Mutated	67	(3.8±2.3)	
RAS			**0.043**
Normal	97	(4.0±2.2)	
Mutated	19	(2.6±2.5)	
PTEN nuclear score			**0.003**
Low	48	(3.9±2.2)	
High	27	(5.3±1.4)	
PTEN cytoplasmic score			0.716
Low	14	(4.2±2.4)	
High	61	(4.4±2.0)	
pS6 intensity score			0.744
Low	33	(4.5±1.8)	
High	42	(4.3±2.3)	

OPNc splice variants in six MTC cases with their corresponding matched non-tumoural tissues, from which frozen samples were available. OPNa isoform expression is significantly higher in the MTC tumour samples than in non-tumoural specimens ($P=0.032$). Conversely, OPNb and OPNc isoforms have similar expression levels in tumour and in non-tumour tissue samples (Fig. 2A). In addition, the expressions of these three splice variants were also analysed in two MTC-derived cell lines (TT and MZ-CRC-1). Both MTC cell lines expressed the three OPN splicing isoforms. Similarly to tumour tissue specimens, OPNa expression levels are higher than OPNb and OPNc expression levels (OPNa > OPNb > OPNc), being the expression higher in TT cell line than in MZ-CRC-1 (Fig. 2B).

Overexpression of OPN isoforms in TT cell line, decrease cell growth and viability

To examine the contributions of each OPN isoform in medullary thyroid cancer, we used *in vitro* gain-of-function experiments. We chose the approach to stable overexpress each of these splicing isoforms in TT cells once this cell line is a well-established model for medullary thyroid cancer functional studies (27). Analysis by real-time PCR demonstrated the overexpression of OPN isoforms in TT cell lines transfected by OPN isoforms, showing higher mRNA levels of the corresponding isoform in relation to their levels in EV-transfected cells (Fig. 3A). The over-expression of OPN protein in TT cell lines transfected with OPNa, OPNb and OPNc isoforms was confirmed through immunocytochemistry analyses using OPN antibody (Fig. 3B). By contrast, OPN protein expression was poorly identified in control cells (EV) (Fig. 3B). Additionally, we evaluated cell proliferation by MTT assay, and we observed that TT cells overexpressing OPNa, OPNb and OPNc significantly reduced growth when compared with control cells (EV) (Fig. 3C). We also confirmed the viability of these cells using Trypan blue exclusion, and we found that OPNa isoform is able to significantly reduce the viability when compared with the control (EV) (Fig. 3D).

Discussion

In this study, we investigated the expression pattern of the glycoprotein OPN in 116 MTCs and correlated it with clinicopathological features of the cases. We observed that OPN expression is a potential marker of good prognosis for MTCs, and we gathered suggestive evidence that total OPN can be a newly identified differentiation marker of (parafollicular) C-cells.

Besides the aforementioned studies (15, 28) in thyroid cancer, OPN expression has been investigated by other groups (29, 30, 31, 32, 33, 34), but only one study evaluated MTC; in this study, Briese *et al.* (31) observed an up-regulation of OPN in three of the four MTCs of their series (75%). Our results, obtained in a much large series, confirm such percentage and demonstrate that 78.4% (91/116) of MTCs express OPN. We have also found that OPN expression is significantly associated with smaller, usually non-invasive cancers. In other words, at variance with PTC, in MTCs, the expression of OPN is associated with features of good prognosis and is reduced during tumour progression.

Interestingly, we observed that isolated C-cells scattered in adjacent normal thyroid parenchyma show strong

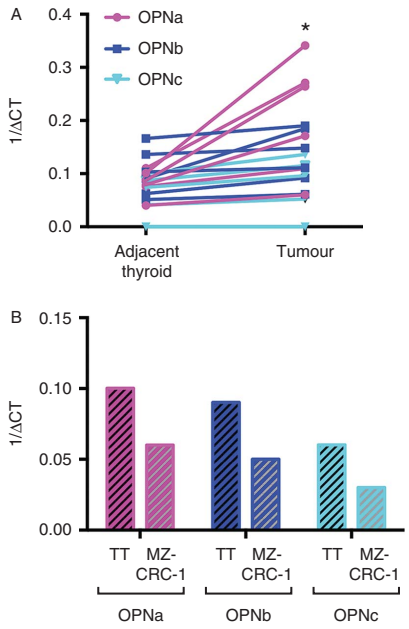

Figure 2

Expression of OPN mRNA splicing isoforms in thyroid tissues and in MTC cell lines. (A) Expression of OPNa, OPNb and OPNc splicing isoforms by qRT-PCR in MTC specimens and in the adjacent thyroid. (B) Expression of OPNa, OPNb and OPNc splicing isoforms in TT and in MZ-CRC-1 cell lines by qRT-PCR. Expression of OPN isoforms is presented relative to *GAPDH* gene expression. A full colour version of this figure is available at http://dx.doi.org/10.1530/EJE-15-0577.

OPN expression. A strong expression of OPN was also observed in clusters of C-cell hyperplasia (both in cases with and without germinal *RET* mutation – data not shown), indicating that this multifunctional protein may be a differentiation marker for C-cells. This assumption is supported by the observation, using serial sections, of strong calcitonin staining in the C-cells displaying OPN expression (Fig. 1F). In contrast to the strong expression in C-cells, we did not observe any OPN expression in follicular cells of the adjacent 'normal' thyroid tissue. Our results suggest that OPN immunostaining may be a useful marker in the study of the physiology and physiopathology of C-cells, C-cell hyperplasia and MTC, but further studies with an in-depth analysis of C-cell characteristics are needed to confirm this assumption.

Often OPN up-regulation in tumours has been associated with poor prognosis (34), but due to its versatility, this protein is also able to play roles that may be related to protective factors and cell differentiation. For instance, OPN expression usually correlates with the intermediate and later stages of osteoblast differentiation from mesenchymal stem cells (35, 36, 37). Furthermore, recent studies support the requirement of OPN expression in maintaining macrophage differentiation and function (38), and Chang *et al.* (39) reported that OPN is implicated in keratinocyte differentiation. Taken together, these findings and our observations suggest the potential role of OPN in differentiation of specific cell types and, in particular, as we are herein advancing, in C-cells.

Using IHC, OPN staining pattern in MTC is consistent with a predominant intracellular localisation of OPN (iOPN). OPN is usually known as an extracellular protein. There is increasing knowledge that extracellular OPN (eOPN) binds to its main receptors, integrins and CD44, activating their corresponding signalling pathways and mediating different OPN functions in cancer cells (40). However, other reports have also described iOPN, both in the cytoplasm and in the nucleus, in distinct cell contexts (41, 42). Similarly to eOPN, iOPN isoforms have also been associated to intracellular CD44 complex (43) and related to cellular processes such as cytoskeletal relocation and cell motility (42, 44). Some authors have suggested furthermore that iOPN and eOPN can act in concert (41, 42). Our results disclose the predominant intracellular expression of OPN in MTC cells. We cannot rule out that an unbalanced proportion of iOPN and eOPN can be present in C-cells and MTC, and that the aforementioned proportion may also modulate tumour- and tissue-specific OPN functional roles (41, 45). Additional studies should be performed to determine the putative regulation and functional roles of both iOPN and eOPN in C-cells and MTC cells.

Although nuclear PTEN function remains poorly understood, it has been shown that acting as a tumour suppressor, nuclear PTEN might lead to cell cycle arrest and decreased tumour growth (46, 47). It was also recently described the maintenance of chromosomal stability as another function of nuclear PTEN (48). PTEN has been related with OPN regulation, and it was reported that *PTEN* overexpression suppresses cellular migration stimulated by OPN (49). As higher levels of OPN were significantly associated with higher levels of nuclear PTEN in MTCs, these two molecules can probably act together in the maintenance of the differentiation of C-cells, thus contributing to prevent tumour progression.

In sporadic MTC, *RAS* mutations are often detected in *HRAS* and *KRAS* genes (25), representing an alternative genetic event to *RET* mutation. Casson *et al.* (50) showed that *RAS* mutation induces expression of OPN in human esophageal cancer, and other authors reported that OPN promoter have target sequences for RAS binding, inducing OPN expression in both human (51) and mouse (52). At variance with the presence of *RET* mutations that were not associated with OPN expression, we observed that *RAS*-mutated MTC cases display a significantly lower expression of OPN. Although in apparent contradiction with the results obtained in other tissue models cited previously (50, 51), our results fit in with the hypothesis that OPN expression is associated with normal differentiation of C-cells. Many studies have demonstrated loss of thyroid-specific gene expression in thyroid cells following the introduction of a variety of transforming oncogenes, in particular of the *RAS* gene family (53). This issue, as far as we are aware, has not been assessed to date concerning C-cell differentiation.

Another interesting result of the present study relies in the demonstration of the differential expression of the three OPN isoforms in MTC. It is known that OPN splicing variants present different and specific roles depending on the tissue and the tumour type (for a thorough review, see reference (54)). While in some tumour models, an individual OPN splicing isoform can act as a tumour-stimulating factor, in other tumour types, the same variant may present the opposite effect, acting against tumour progression (24, 54, 55, 56). We observed that total OPN, detected by IHC with an antibody that recognises all three OPN isoforms, is expressed in MTCs. Unfortunately, the expression of the different isoforms in paraffin sections could not be evaluated for technical reasons. We were able to evaluate the mRNA expression of OPN isoforms, by real-time PCR, using distinct primers to recognise three different isoforms, in six pairs of tumours and respective adjacent thyroid parenchyma. To the best of our knowledge, this is the first study reporting the mRNA expression of OPN isoforms in MTCs. OPNa mRNA

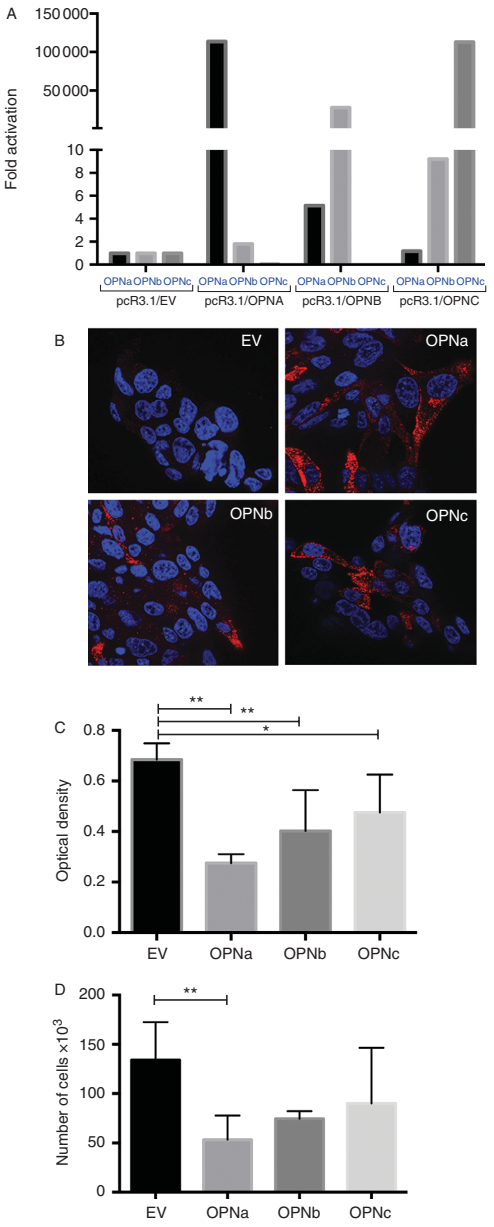

Figure 3

Proliferation and viability in TT cells overexpressing OPNa, OPNb, OPNc and EV. (A) Real-time PCR showing relative expression in TT cells overexpressing OPNa, OPNb, OPNc and EV. (B) Immunocytochemistry analyses of OPN expression in control cells (TT cells with EV) and in TT cells overexpressing OPNa, OPNb and OPNc. (C) MTT and (D) Trypan blue assays showing significantly decreased proliferation levels in TT cells over-expressing OPNa, OPNb, OPNc and EV ($n \geq 3$; $^{*}P < 0.05$; $^{**}P < 0.005$). A full colour version of this figure is available at http://dx.doi.org/10.1530/EJE-15-0577.

was expressed at significantly higher levels in tumour samples than in non-tumour samples ($P=0.032$), while OPNb and OPNc presented similar levels of mRNA expression in tumours and respective adjacent tissues. We realised that when analysing the so-called 'normal adjacent thyroid', we were evaluating mainly follicular cells and comparing the expression of the isoforms between follicular cells and MTC. Only by analysing isolated C-cells we were able to compare "normal" C-cells and neoplastic MTC cells. Nevertheless, as we observed that OPNb and OPNc had similar expression levels in 'normal' adjacent tissue and in MTC, whereas OPNa showed significantly higher levels in MTC, we can conclude that OPNa mRNA is the isoform more expressed in MTC. This conclusion fits with our observation of higher levels of OPNa mRNA in the two MTC-derived cell lines. It remains to be clarified whether the same is true regarding normal and hyperplastic C-cells, i.e. if OPNa mRNA high expression is also present in normal and hyperplastic C-cells, or if it is tumour specific.

Additionally, we examined the functional roles of each OPN splicing isoform in medullary thyroid cancer progression by using an *in vitro* model. Here we show that TT cells overexpressing OPNa, OPNb and OPNc show a significant decrease in proliferation and viability, being OPNa isoform having the most prominent effect. Although OPN is widely known as implicated in promoting invasive and metastatic progression in many carcinomas (11, 57), in medullary thyroid cancer, the overexpression of OPN isoforms seems to be related with a protective role, as reported in pancreatic adenocarcinoma (58). These results indicate that in MTC, OPN contributes to impair tumour growth, corroborating the hypothesis that OPN can act in the maintenance of the differentiation of C-cells. Further studies, including evaluation of adhesion, invasion and migration, would allow a view of the whole picture and the complete understanding of the role of OPN in C-cell biology and disease.

In summary, the present study shows for the first time that OPN is expressed in MTCs and that such expression is associated with smaller tumour size, less invasive features and overexpression of nuclear *PTEN*. Yet, our study reports the first description of OPN expression in the specialised neuroendocrine cells of the thyroid, indicating a role for this multifunctional protein in C-cell differentiation. Furthermore, we showed that TT cells overexpressing OPNa isoform show reduced proliferation and viability, indicating that OPN contributes to prevent tumour progression.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Chapter 6 – General Discussion and Conclusions

Our study on the role of OPN and its variants revealed the broad spectrum of functions that this protein can have inside a tissue. This study shows that in thyroid, in different kind of cells, OPN can have diverse effects, acting as a promoter of tumour aggressiveness in follicular-tumours derived cells, and as a differentiation marker in parafollicular cells.

OPN has been widely studied since its discovery in 1979 [1]. Early works reported it as a phosphoprotein in osteoblasts secreted to the bone extracellular matrix [3]. Then many functions have been attributed to this molecule, involved in both physiological and pathologic events, such as biomineralization, bone resorption, cell adhesion, migration and cell survival, angiogenesis, apoptosis, inflammation and wound healing [45].

When we analysed the OPN protein expression in tumours derived from follicular cells, we found that tOPN is predominantly expressed in cPTC, when compared to adjacent non-tumoural thyroid tissues, and significantly associates with hyaline stroma and vascular invasion. These data are in accordance with OPN as a glycoprotein secreted in the ECM, both in tumour and non-tumoural tissues [45]. OPN is also known to be upregulated in the tissue stroma in different conditions, such as salivary pleomorphic adenomas [199] and intrahepatic cholangiocarcinoma [200]. It is well known that the interplay between epithelial cells and the microenvironment may contribute to keep the epithelial polarity and to modulate growth inhibition [201]. On the other hand, the stromal compartments undergo changes in response to emerging epithelial lesions that can have a key role in cancer initiation and progression [201, 202]. Accordingly, our data regarding the correlation of tOPN and the presence of a hyaline stroma support the assumption that either tOPN expression alone or tOPN in association to hyaline stroma may play a role in

cPTC tumour aggressiveness. Of note, cPTC samples displaying vascular invasion exhibited higher tOPN staining scores than tumours without vascular invasion. Such results also corroborate previous studies which showed that tOPN overexpression is significantly associated with poor prognostic factors, such as presence of lymph node metastasis, tumour size and poor disease free survival in PTC samples [100, 102, 103, 203].

At variance with the aforementioned results, and the majority of published papers which OPN functions are related to malignancy and tumour progression, we described OPN as a differentiation marker for normal C-cells in thyroid, whereas in medullary thyroid cancer, OPN appear associated with good prognosis. Parafollicular cells are cells with neuronal differentiation, and in this cells OPN is expressed and appear as a differentiation marker. Similarly, Melen-Mucha *et al*. [204] reported that OPN levels were decreased in Gastro-entero-pancreatic/neuroendocrine (NET) tumours, when compared with healthy controls. Moreover, a recent study showed that OPN deficient mouse ($OPN^{-/-}$) crossed with TRansgenic Adenocarcinoma of the Mouse Prostate (TRAMP), developed aggressive human-like neuroendocrine cancer, instead of prostate carcinoma [205]. These data shed light to the role of OPN in the different cancer types, thus its expression seems to protect against the development of aggressive neuroendocrine tumours.

Regarding expression of OPN in MTC, our series represents the largest reported to date. We demonstrate that 78.4% (91/116) of MTCs express OPN and such expression is significantly associated with smaller, usually non-invasive tumours. Therefore, on the contrary of what is frequently found, the expression of OPN in MTC is associated with features of good prognosis and is reduced during tumour progression. During evaluation of the slides, we also observed strong OPN expression in isolated C-cells scattered in

adjacent normal thyroid parenchyma, and strong OPN expression was also observed in clusters of C-cell hyperplasia. Together, these results indicate that OPN may be a differentiation marker for C-cells. This assumption is supported by the observation of strong OPN expression in C-cells, confirmed by strong calcitonin staining in the same tissues (see paper III, Fig. 1F). In contrast to the strong expression in C-cells, we did not observe any OPN expression in follicular cells of the adjacent 'normal' thyroid tissue.

Additionally, we also found that high levels of OPN protein expression in MTC was correlated with high levels of nuclear PTEN expression. Although nuclear PTEN function remains poorly understood, it has been shown that acting as a tumour suppressor, nuclear PTEN might lead to cell cycle arrest and decreased tumour growth [206, 207]. It was also recently described the maintenance of chromosomal stability as another function of nuclear PTEN [208]. PTEN has been related with OPN regulation, and it was reported that PTEN overexpression suppresses cellular migration stimulated by OPN [209]. As higher levels of OPN were significantly associated with higher levels of nuclear PTEN in MTCs, these two molecules can probably act together in the maintenance of the differentiation of C-cells, thus contributing to prevent tumour progression.

At variance with the presence of RET mutations that were not associated with OPN expression, we observed that RAS-mutated MTC cases display a significantly lower expression of OPN. Although in apparent contradiction with the results obtained in other tissue models cited previously [119, 210], our results fit in with the hypothesis that OPN expression is associated with normal differentiation of C-cells. Many studies have demonstrated loss of thyroid-specific gene expression in thyroid cells following the introduction of a variety of transforming oncogenes, in particular of the RAS gene family [211]. This issue, as far as we are aware, has not been assessed to date concerning C-cell differentiation.

Therefore, concerning tOPN protein expression in the tumour types derived from different cells in thyroid, and its association with clinicopathological data, we found opposite results thus leading to opposite consequences when we analyse follicular and parafollicular cells in thyroid.

We then evaluated the transcript expression of tOPN and the three OPN-SV in both models (follicular and parafollicular -derived cells in thyroid). Regarding the follicular-derived cells, we found that among the three OPN-SV, OPNa is also overexpressed in all thyroid tissues (adjacent thyroid tissue, adenoma, FTC, fvPTC and cPTC), and that among such different tissues, OPNa is overexpressed in cPTC. Furthermore, we found that the OPNa overexpression is specifically associated with poor prognostic features in cPTC. In particular, OPNa variant was significantly associated with presence of extrathyroid extension, vascular invasion and $BRAF^{V600E}$ mutation. In the fvPTC, the only significant association observed was between high tOPN and OPNa expression levels and older patients. Based on these findings, we postulate that elevated OPNa expression in TC may occur during tumour progression, facilitating more aggressive phenotypes. Other studies have shown that OPN-SV expression and its associations to clinicopathological features seem to be tissue specific. For instance, it has been reported that high OPNb and OPNc expression levels in breast cancer [212] and in gastric tumours [213] is correlated with more aggressive clinicopathological features.

In parafollicular-derived cells, we investigated the expression in 6 MTC cases and in 2 cell lines (TT and MZ-CRC1). OPNa mRNA was expressed at significantly higher levels in tumour samples than in non-tumour samples ($P = 0.032$), while OPNb and OPNc presented similar levels of mRNA expression in tumours and respective adjacent tissues. We realised that when analysing the so-called ‘normal adjacent thyroid’, we were evaluating mainly follicular cells and comparing the expression of the variants between

follicular cells and MTC. Only by analysing isolated C-cells we were able to compare "normal" C-cells and neoplastic MTC cells. Nevertheless, as we observed that OPNb and OPNc had similar expression levels in 'normal' adjacent tissue and in MTC, whereas OPNa showed significantly higher levels in MTC, we can conclude that OPNa mRNA is the variant more expressed in MTC. This conclusion fits with our observation of higher levels of OPNa mRNA in the two MTC-derived cell lines. It remains to be clarified whether the same is true regarding normal and hyperplastic C-cells, i.e. if OPNa mRNA high expression is also present in normal and hyperplastic C-cells, or if it is tumour specific.

In order to disclose the possible roles of OPNa and/or the two other OPN-SV, we chose three thyroid tumour-derived cell lines (two from follicular cells, c643 and 8505c; and one from parafollicular MTC, TT). We overexpressed OPNa, OPNb or OPNc in c643 and 8505c cell lines (follicular-derived TC cells) with plasmid constructs containing each of the three OPN- SV. Then, we observed that follicular-derived TC cells overexpressing OPNa displayed significantly increased cell growth, migration and motility, whereas OPNb and OPNc overexpression in TC cells did not induce similar effects. Other reports have demonstrated that different OPN-SV play important roles in tumour progression by regulating cell growth, adhesion, migration and tumour formation. Our group as previously demonstrated in prostate and ovarian carcinoma model that OPNc activates invasion and adhesion properties, as well as metastatic potential and angiogenesis [30, 31]. Others demonstrated that in hepatocellular carcinoma cells, OPNa and OPNb can induce cell migration [37]. Lin J and co-workers in cells derived from esophageal adenocarcinoma [214], observed that OPNb overexpression evoked enhanced cell proliferation, migration and invasion. Our findings highlight the specific importance of

OPNa in follicular cells derived-TC promoting cell growth and migratory and invasive phenotype.

Hence, to further explore OPNa roles on modulating TC invasive properties in such TC follicular derived cell lines, we also explored the role of OPNa in the activation of MMPs. MMPs are important enzymes in the metastatic cell arsenal. These proteins can degrade both cell adhesion molecules and extracellular matrix molecules, enabling tumour cells to migrate from the tumour bulk and to invade adjacent tissues [215]. Previous data from our group in prostate and ovarian tumour models showed that cells overexpressing the OPNc variant induce expression of MMP2 and MMP9, highlighting the functional tissue specificity of OPN-SV [31]. Thyroid carcinomas produce elevated levels of MMP2, which has been correlated with the presence of lymph node metastasis [29]. Herein, we have found that CM collected from cells overexpressing OPNa have increased activity of MMP2 in c643 cells and MMP2 and MMP9 in 8505c cell lines. These results evidence that OPNa may promote TC cell invasion through inducing MMP2 and MMP9 secretion. The detailed mechanism by which this event occurs still needs further characterization. Nonetheless, it has been described that OPN can regulate MMPs activity by binding to pro- MMP9, thus promoting its activation [216]. Additionally, OPN can induce NFkB-mediated pro-MMP2 and MMP9 activation through IkBa/IKK signalling pathway [217].

Taking in consideration that MMPs expression and cancer cell migration are fundamental features for tumour invasion [218], we further investigated the contribution of OPNa variant for a TC cell line invasiveness using an *in vivo* experimental model. Using the CAM assay approach, we observed that tumours formed by the overexpressing OPNa cells present a loosen structure, in which the cells were oriented towards the invasion front while single cells and cell clusters invading the CAM mesenchyme could

also be observed. In contrast to this, EV clones formed compact tumours, with clear de ned boarders lacking invading cells. Similarly to our data, overexpression of OPNa has been previously associated with modulation of cancer cell invasion in mesothelioma, breast cancer and hepatocellular carcinomas [33, 37, 219]. Although activating cell invasion, OPNa overexpression does not significantly modulated angiogenesis and tumorigenesis in this *in vivo* tumour model. Some oncogenic proteins can activate some specific steps in tumour progression, but not others. As evidenced by our data OPNa overexpression *in vivo* may predominantly modulate signalling pathways that stimulate migration and invasion, possibly through stimulating ECM degradation by MMP2 and MMP9. In light of our results, we may hypothesize that the increased invasive capacity of c643 cells overexpressing OPNa (in comparison to c643-EV cells) allows the cells to reach the blood vessels, and have no effect in the recruitment of new vessels.

Respecting to the parafollicular-derived cells, and in contrast of what we observed for follicular-derived cells, we show that TT cells overexpressing OPNa, OPNb and OPNc show a significant decrease in proliferation and viability, having OPNa variant the most prominent effect. Although OPN is widely known as implicated in promoting invasive and metastatic progression in many carcinomas [31, 220], in MTC, the overexpression of OPN variants seems to be related with a protective role, as reported in pancreatic adenocarcinoma [221]. These results indicate that in MTC, OPN contributes to impair tumour growth, corroborating our hypothesis that OPN can act in the maintenance of the differentiation of C-cells. Further studies, including evaluation of adhesion, invasion and migration, would allow a view of the whole picture and the complete understanding of the role of OPN in C-cell biology and disease.

Although is widely established the deep relationship between OPN and calcification, little is known regarding the OPN expression and the psammoma bodies

(PB) formation in cPTC cases. Thus, to better comprehension of such process, we intended to explore this issue. For that, we investigate the relationship between the expression pattern of the OPN and OPN-SV in cPTC cases and correlated it with the occurrence of PB in such cases. Moreover, we analyse if the presence of PB in cPTC cases is related with its clinicopathological data. We observed that high expression of OPNa transcript is correlated with PB in cPTC cases. We also found that cPTC cases having PB are associated with younger patients and presence of lymph node metastasis. Moreover, OPNa overexpression displayed a strong capacity to promote calcification and substantial collagen synthesis in the thyroid cancer cell lines.

We have shown that tOPN protein expression has been observed in cPTC samples that present PB. Of note, in the immunohistochemistry analysis of the cases stained for tOPN, PB are much clearer than in HE staining, particularly since the staining of PB with anti-OPN antibody is stronger. To date, only two studies reported an association between OPN expression and the occurrence of PB in PTC samples [85, 86]. Both studies showed that the expression of tOPN protein associates with the occurrence of PB in PTC samples, suggesting a role for OPN in calcification of PTC [85]. Another association between OPN and calcification in cancer was reported by Hirota *et al*. [222], who described OPN protein co-localization with calcium phosphate in meningioma tissues, further evidencing a role for OPN on the PB formation in this context.

We further showed that cPTC cases that presented PB also had higher expression levels of tOPN, OPNa, OPNb and OPNc transcripts, although only OPNa and OPNb levels demonstrated statistically significant differences (OPNa > OPNb). In a previous work, we demonstrated that among the three OPN-SV, OPNa had the highest expression levels in cPTC, when compared with other thyroid tissues [223]. Therefore, the current results are in accordance with our earlier observations, raising a possible relation between

OPNa variant overexpression and the formation of PB in cPTC. Regarding the small series of diffuse sclerosing PTC samples analysed in this study, no expression of tOPN protein was observed. On the other hand, when we evaluated the three OPN-SV in these cases, high OPNa levels have been detected. In addition to the cPTC, the diffuse sclerosing PTC is the variant that frequently presents numerous PB [224], as also confirmed in the 3 cases analysed here. Notably, diffuse sclerosing PTC have been described to have a higher incidence of lymph node metastasis when compared to cPTCs [225].

Regarding the analysis of cPTC clinicopathological features, we found a correlation between cPTC cases presenting PB and presence of lymph node metastasis and younger patients. PB is a diagnostic indicator for cPTC, and its presence strongly suggest tumour malignancy in preoperative diagnosis. Bai *et al.* [226] also reported that the presence of PB in PTC cases was associated with gross lymph node metastasis and high-stage cancer (stage IVa). Conversely, Pyo *et al*. [227] found an association between PB and tumour multifocality, extrathyroid extension, and lymph node metastasis. The higher expression of OPNa in cPTC containing PB and the association of PB with lymph node metastases is in accordance with our previous data, in which we showed that OPNa overexpression contributes to cPTC progression features [223]. Bai *et al*. [226] found an association between stromal calcification and advanced age of patients (> 60 years). However, in that study authors only considered calcification in the stroma. Stromal calcification frequently arises in benign lesions, whereas PB are suggestive of malignancy. Herein, we evaluated only PB in cPTC cases, excluding other dystrophic or stromal calcifications, as well as bone formation. In fact, when we consider dystrophic calcification, no correlation was found with OPNa expression (*data not shown*).

In our previous work, we also reported an association between tOPN protein expression and presence of stromal tissue areas in cPTC cases, suggesting that this may be correlated with tumour aggressiveness in cPTCs [223]. Herein, we further investigated this point and presented *in vitro* and *in vivo* evidence of an association particularly between OPNa expression and the presence of PB and stromal collagen.

In order to establish putative impacts of OPN-SV regarding the calcification process, OPNa, OPNb and OPNc (and an empty vector as a control) were overexpressed in c643 TC cells. We then analysed the effects of OPN-SV overexpression in the formation of calcium deposits in the ECM produced by the cells and found that OPNa overexpressing cells strongly induced calcium deposits in the ECM. Calcification within the thyroid gland is a common finding both on thyroid imaging and thyroid histopathologic findings [228]. Although PB are a highly specific sign of malignancy frequently detected in cPTC [227], the mechanisms by which PB are formed in thyroid tumours is still controversial. Several reports have revealed that calcification is more common in malignant than in benign thyroid nodules [229, 230]. Other authors reported that intrathyroidal calcification was noted in 26.1% (29 out of 111) of the malignant thyroid nodules and in only 8.0% (20 out of 250) of benign thyroid samples [228]. Nonetheless, it is important to mention that many authors have been stressing that the presence of intrathyroidal calcification *per se* cannot be used to distinguish between benign and malignant thyroid disease [229-232].

We also observed that, among the overexpressing OPN-SV c643 cells, OPNa overexpressing cells induced more efficiently collagen synthesis in the ECM. This data indicates that among the three variants, OPNa remarkably promoted these effects. These results are in accordance with our previous observations, whereby OPNa (among all OPN-SV) seemed to be the key OPN variant associated with the presence of stroma [223].

The roles of OPN in the tumour ECM microenvironment has been investigated. For instance, Liaw *et al*. [233] showed that OPN knockout mice exhibit defective repair of incisional skin wounds with abnormal collagen fibrillogenesis. Additionally, it is known that depending on the ECM stiffness, collagen fibbers potentiate cell migration, especially in the presence of MMPs activity [234]. Accordingly, our previous work demonstrated that OPNa activates MMP2 and MMP9 activities in thyroid cell lines, besides promoting cell migration and invasion in the *in vivo* CAM model [223]. Being the most abundant ECM components, collagens constitute up to 90% of the ECM and 30% of the total proteins in humans, also providing the structural integrity and the tensile strength of human tissues and organs [235]. Furthermore, the increased collagen deposition is the most well-recognized ECM alteration in tumour tissue [236-238]. In the context of cancer biology, it has also been shown that collagens regulate the physical and the biochemical properties of tumour microenvironment, modulating cancer cell polarity, migration and signalling [239-241].

In order to evaluate OPNa properties on inducing calcium deposit and collagen synthesis in the ECM, we selected TPC1 among nine thyroid cell lines, due to its highest levels of endogen OPNa expression. Similarly to what has been observed for c643 cells overexpressing OPNa variant, TPC1 cells also exhibited calcium deposits and production of collagen fibbers. TPC1 cell line is recognized as presenting the *RET/PTC* rearrangements [242]. This molecular alteration was demonstrated to induce OPN expression in thyroid cell lines, such as PCCl3 [99], reinforcing the hypothesis that OPN plays a role in such processes. Although our results also provide evidence in the same direction, in particular giving some early evidence that OPNa plays a role in such mechanism, further studies are needed to better understand how OPNa modulates collagen synthesis and the calcification process. In addition, it is imperative to better

comprehend the underlying molecular mechanisms that dictate the lymphovascular invasion, thus contributing to the pathophysiology knowledge of cPTCs.

Our results show that the presence of PB is associated with lymph node metastases in cPTC samples. Additionally, OPNa overexpression and its association with the occurrence of PB in cPTC samples, together with its strong capacity to promote calcium deposit and to synthesize collagen in the ECM in thyroid cells *in vitro*, supports an important role for OPNa in the etiopathogenesis of the cPTC.

In summary, our data demonstrated that OPNa is the prevalent OPN-SV in DTC tissues and cell lines, and that overexpression of OPNa is associated with poor prognostic and invasive features in PTC. Yet, OPNa overexpression in TC cell lines strongly increases cell migration, invasion and MMPs activity, evidencing a major role for OPNa in TC progression features.

The present study also showed the overexpression of OPNa and its association with the occurrence of PB in cPTC samples, together with its strong capacity to induce calcium deposit and to synthesize collagen in ECM in thyroid cells *in vitro*, suggest a role for this molecule on the physiopathology of the cPTC.

Finally, this Thesis demonstrated for the first time that OPN is expressed in MTCs and that such expression is associated with smaller tumour size, less invasive features and overexpression of nuclear PTEN. Yet, our study reports the first description of OPN expression in the specialised neuroendocrine cells of the thyroid, indicating a role for this multifunctional protein in C-cell differentiation. Furthermore, we showed that TT cells overexpressing OPNa variant show reduced proliferation and viability, indicating that OPN contributes to prevent tumour progression. These data suggest that OPN immunostaining may be a useful marker in the study of the physiology and

physiopathology of C-cells, C-cell hyperplasia and MTC, although further studies with an in-depth analysis of C-cell characteristics are needed to confirm this assumption.

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Chapter 7 – Appendix

7.1 – Additional publications in the PhD time course

7.1.1 Paper I - Poorly differentiated and undifferentiated thyroid carcinomas

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Poorly Differentiated and Undifferentiated Thyroid Carcinomas

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ABSTRACT

Thyroid cancer is the most common endocrine malignancy and its incidence goes on increasing worldwide. The majority of thyroid tumours comprise well-differentiated (papillary and follicular) thyroid carcinomas that usually carry an excellent prognosis, while a minority progress to poorly differentiated carcinoma (PDTC) and, ultimately, to the highly aggressive and lethal undifferentiated carcinoma (UTC). Recently, some major advances have been made on the histologic and imunohistochemical identification, as well as on the molecular characterization of PDTC and UTC. In this review we summarize the most recent immunohistochemical and molecular findings in PDTC and UTC, giving a particular emphasis to the diagnostic and prognostic meaning of the genetic alterations.

Key Words: Anaplastic thyroid carcinoma, Non-medullary thyroid carcinoma, Molecular medicine, Small cell carcinomas, Poorly differentiated thyroid carcinoma

INTRODUCTION

The most important difference between the classifications of thyroid tumours in the WHO books of 1988 and 2004 concerns the individualization in the latter of the group of poorly differentiated carcinomas (PDTC) (1, 2). It was also recognized that all sorts of benign and malignant thyroid tumours may be composed by the individualization of oncocytic (oxyphilic or Hürthle) cells, thus leading to oncocytic/Hürthle cell variants of adenoma and of follicular papillary and poorly differentiated thyroid carcinoma (1). It is usually advanced that the absence of an oncocytic/Hürthle cell variant of undifferentiated carcinoma (UTC) reflects the high mitotic ratio of such tumours (the cells of undifferentiated/anaplastic carcinoma divide too fast to allow the accumulation of mitochondria in their cytoplasm) (3).

The immunohistochemical and molecular characteristics of poorly differentiated and undifferentiated carcinomas were thoroughly addressed in the 2004 edition of the WHO book, as well as in a number of review papers by Garcia-Rostan et al., Nikiforov et al. (4-6) and Soares et al. (7) (Tables 1, 2).

Most of the problems found in the stratification of such immunohistochemical and/or molecular markers reflect differential diagnostic difficulties – it is not easy, for instance, to separate a poorly differentiated carcinoma from a widely invasive follicular carcinoma with a trabecular/solid growth pattern – and the intrinsic heterogeneity of both groups of carcinoma.

Another problem regards the existence of several clinico-pathological entities in thyroid oncology that may be considered as a sort of poorly differentiated carcinoma [e.g. (sclerosing) mucoepidermoid carcinoma and mucinous carcinoma)]. Such entities have been recently described and discussed in the 2014 - AFIP book on Thyroid and Parathyroid Tumours (8) and will not be addressed in the present review. The only exception concerns the group of Small cell carcinomas/Carcinoma of the thyroid with Ewing Family Tumour Elements (CEFTE) because it represents a particularly interesting end result of new developments in the immunohistochemical and molecular study of peculiar thyroid tumour that raise difficult differential diagnostic problems (9-12).

POORLY DIFFERENTIATED CARCINOMA

The histological description should follow the 2004 WHO book and the AFIP book of Rosai et al. (1, 8) (Figure 1A-E). The diagnostic guidelines and the histological pictures in the latter are excellent and review thoroughly the steps used in the diagnosis of PDTC following the algorithmic approach advanced by Volante et al. (13).

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Table I: Poorly differentiated carcinoma

	Antibodies usually expressed	Antibodies variably expressed	Antibodies usually not expressed
Immunohistochemical features	Cytokeratins TTF1 Thyroglobulin (focal) Ki-67 (labelling index is higher than in well differentiated carcinomas) Cyclin D1	Synaptophysin p53 E-cadherin	Calcitonin Chromogranin
Molecular features	**Molecular alterations**	**Prevalence (%)**	**References**
	RAS	20-50	(7); (4)
	TP53	15-40	(7); (4)
	BRAF	5-20	(7); (4); (31)
	CTNNB1	5-25	(31); (28)
	PIK3CA	5-14	(7); (4); (31)
	PTEN	20	(31)
	AKT1	5-10	(4)
	TERT	20-50	(7); (36)
	STRN/ALK	9	(42)

PDTC: Immunohistochemical and molecular features (poorly differentiated carcinomas).

Table II: Undifferentiated thyroid carcinoma

	Antibodies usually expressed	Antibodies variably expressed	Antibodies usually not expressed
Immunohistochemical features	Cytokeratins (usually focal) Ki-67 (labelling index is higher than in PDTC) p53 Vimentin Cyclin D1	TTF1 PAX8 E-cadherin EMA P63 CEA	Calcitonin Chromogranin Thyroglobulin Synaptophysin Bcl-2 CD31 CD34
Molecular features	**Molecular alterations**	**Prevalence (%)**	**References**
	TP53	50-80	(19); (4)
	TERT promoter mutation	30-50	(19);(56)
	RAS	4-60	(49)
	BRAF	10-50	(19);(4)
	CTNNB1	5-66	(29)
	PI3KCA	5-25	(19)
	PTEN	5-15	(19); (4)
	AKT1	5-10	(19); (4)
	ALK	0-10	(19)

UTC: Immunohistochemical and molecular features (undifferentiated carcinoma).

The most difficult diagnostic problems occur in the border between well differentiated (WDTC) and poorly differentiated carcinoma. The separation between papillary thyroid carcinoma (PTC) and PDTC is based upon the nuclear features of the neoplastic cells. In case the nuclei are of the PTC type the tumour is classified as a solid or trabecular variant of PTC regardless of the existence of necrotic foci and/or high mitotic number (6, 14, 15).

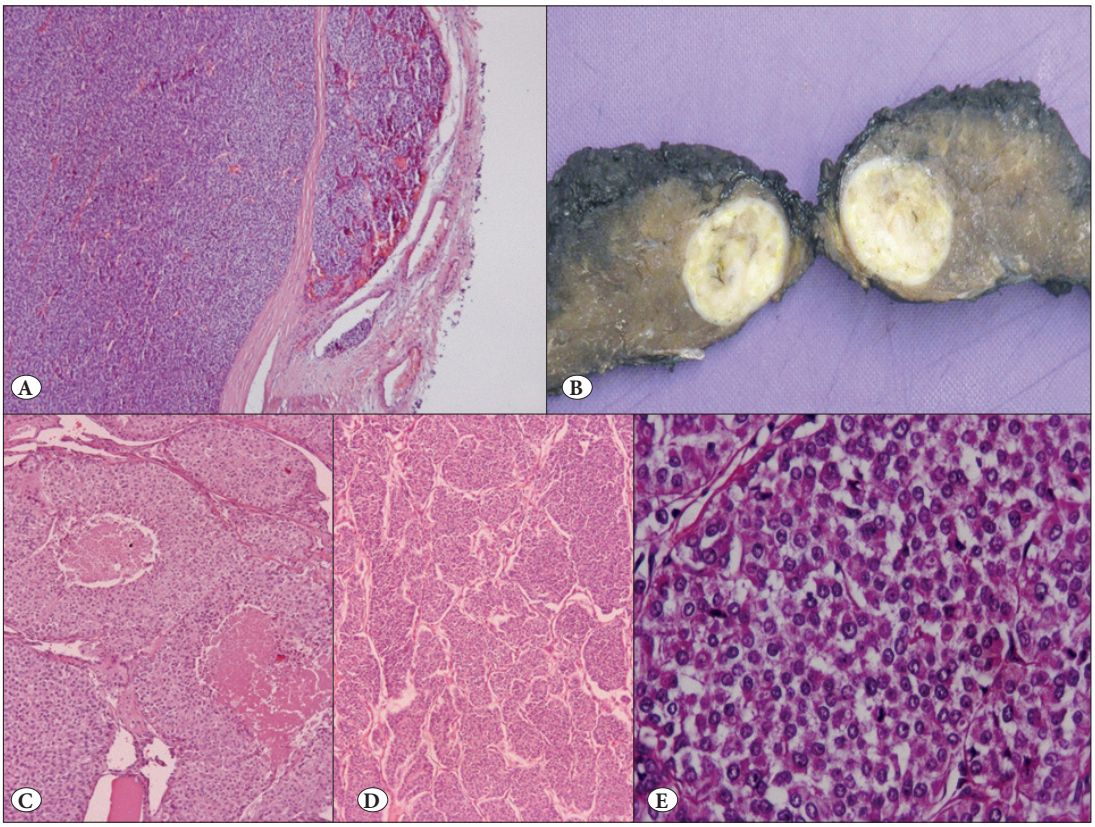


Figure 1: Poorly Differentiated Thyroid Carcinoma (PDTC): A) Tumour with expansive growth pattern, invasion of the capsule and images of vascular invasion, **B)** Macroscopic appearance of an apparently well circumscribed PDTC whose histological characteristics are documented below, **C, D)** Nested pattern area with foci of necrosis (H&E, 40x), **E)** Focus with numerous mitoses and without PTC type nuclei (H&E, 400x).

The separation between widely invasive follicular thyroid carcinomas (FTC) with foci of necrosis and/or high mitotic index and PDTC is almost impossible to establish in many situations (15). Molecular data did not provide so far any diagnostic clue and the pathology reports are written assuming the aforementioned limitations. This does not constitute a major drawback since the prognosis and treatments are similar in both situations (1, 8, 15).

In different fields of well differentiated thyroid carcinomas there are reports of tumours displaying poorly differentiated features [e.g. cribriform morular variant of PTC (16)]. These cases should not be lumped together with common PDTC.

The differential diagnosis between PDTC and UTC/anaplastic carcinoma may be difficult in intermediate cases but is based upon a very precise immunohistochemical feature: PDTC exhibits diffuse nuclear positivity for TTF1 and focal positivity, frequently with a dot-like pattern, for thyroglobulin, and UTC by definition are thyroglobulin negative and almost always TTF1 negative.

The hottest topic in the world of PDTC regards the diagnosis of PDTC, which do not exhibit unequivocal signs of capsular and/or vascular invasion. The question is simple but the answer is difficult. Which are the criteria for diagnosing a PDTC in an apparently non-invasive thyroid tumour? There are anecdotic reports in the literature claiming that such cases exist and have given rise to metastases (17). Ghossein et al. (18) did not find evidence supporting this claim and we must confess we do not know how to solve the problem. In real life, and whenever the

tumour displays (very) aggressive histological features without showing concomitant signs of invasion, we sample the capsule completely and look at deeper sections of the tumour in an attempt to find such signs. The only molecular feature which might be used for making a diagnosis of noninvasive PDTC would be the demonstration of clonal TP53 mutations or diffuse nuclear immunoreactivity for p53, but even then the classification of noninvasive PDTC is questionable (we must confess we have never made such diagnosis).

In the future, there will hopefully exist drugs that can be efficiently used to treat patients with thyroid carcinomas not responding to radioactive iodine, in these settings the focus will rest on the identification of specific molecular targets rather than on the histological classification of the carcinomas.

The molecular features of PDTC are summarized in Table 1. Activating point mutations of the *RAS* gene typically affect codons 12, 13 and 61. At variance with differentiated thyroid cancer where *NRAS* codon 61 mutations are the most frequent, *HRAS* and *KRAS* codon 12 and 13 are also found mutated in PDTC (4, 7, 19). Volante et al. reported *RAS* mutations as the most common genetic alteration in PDTC (20). Moreover, the detection of *RAS* mutations appears to be clinically relevant in terms of prognosis, identifying a subset of more aggressive tumours (20). It was shown that PDTC cases with mutated *NRAS* are significantly associated with the appearance of haematogenous (particularly bone) metastases (19). Due to the association found between *RAS* mutations and guarded prognosis in PDTC and UTC, Wang et al. proposed that a particular attention should also be paid to WDTC, namely FTC, harbouring *RAS* mutations (7, 21).

TP53 gene, encoding a nuclear transcription factor that is typically involved in the negative regulation of the cell cycle and in promoting apoptosis, is a frequently impaired gene during thyroid tumour dedifferentiation (22-24). In thyroid, *TP53* shows a mutational pattern located in known hotspots (exons 5-9) in 15-40% of cases. Moreover, there is a relatively good correlation between mutations and p53 immunohistochemical reactivity: aberrant p53 immunoreactivity is detectable in 40-50% of PDTC (1, 7, 19). *TP53* mutation seems to be (one of) the dedifferentiation switches necessary for progression and dedifferentiation in thyroid tumours (7).

$BRAF^{V600E}$ mutation occurs in 5-20% of PDTC (25, 26). Many of these carcinomas also reveal areas of PTC, and $BRAF^{V600E}$ is present in both tumour components, thus suggesting that this mutation is an early event that do not impair tumour dedifferentiation (4). In accordance with a stepwise progression model, BRAF mutations are almost exclusively found in PDTC arising from PTC, being extremely rare in PDTC associated with FTC (7). Ricarte-Filho et al. (27) reported that 39% of PDTC, FDG-PET positive tumours refractory to radioactive iodine (RAI) treatment harbouring *BRAF* mutations, whereas non-RAI-refractory PDTCs display a significantly lower prevalence of *BRAF* mutations (12%) (27). These results indicate that *BRAF* mutated PDTC are more often refractory to RAI treatment than *BRAF* wild type PDTC.

Two of the most common rearrangements in WDTC are RET/PTC and *PAX8/PPARγ* (4); however, these specific rearrangements are only rarely detected in PDTC (19). This finding suggests that WDTC harbouring such rearrangements do not usually evolve toward less differentiated carcinomas, thus reinforcing the assumption that the majority of "rearranged" PTC and FTC cases do not tend to progress towards further advanced steps of neoplastic development (19).

Another gene reported as frequently mutated in PDTC and UTC is *CTNNB1*, which encodes βcatenin that is involved in cell adhesion and WNT signalling (4). The seminal paper of Garcia-Rostan et al. (28) showed a frequency of 25% of *CTNNB1* mutations in PDTC. The activating mutations cluster in exon 3 at the phosphorylation sites for ubiquitination and degradation of β-catenin and are associated with aberrant nuclear immunoreactivity, suggesting WNT pathway activation (28, 29). In contrast Rocha et al. (30) did not find mutations in *CTNNB1* nor in the *CDH1* gene (encoding E-cadherin) in a series of PDTC. Rocha et al. observed alterations of protein expression, concluding that loss of E-cadherin rather than *CTNNB1* mutation appears to be the crucial event in determining the degree of differentiation of thyroid carcinomas (19, 30). In accordance with these latter results, Pita et al. (31) found *CTNNB1* mutations in a minority (5%) of PDTCs.

Thyroid carcinoma dedifferentiation involves also the progressive accumulation of other mutations, particularly those in genes that encode effectors of the PI3K–AKT pathway, such as the *PIK3CA* (the gene that encodes PI3K), *AKT1* and *PTEN* (4). Among PDTC carcinomas, 5-14% harbour mutations in *PIK3CA*, 20% in *PTEN* and 5–10% in *AKT1* (4, 8, 31, 32).

Telomerase activation is known to be a hallmark of cancer being detected in up to 80% of malignant tumours (7). Normal thyroid tissue is thought to be telomerase

negative, thus raising the possibility that the reactivation of telomerase may be a useful marker of tumour development (33, 34). *TERT* promoter mutations were found in 20-50% of PDTC (8, 33, 35, 36). These findings concur with the evidences that *TERT* promoter mutations associates with a more aggressive behaviour of thyroid tumours (37, 38).

Some PDTC and every UTC constitute the majority of clinically aggressive tumours that cause the death of the patients. In an attempt to address this lethality, efforts have been made for identify other genetic alterations in less differentiated and undifferentiated thyroid carcinomas that could pinpoint new therapeutic targets. Analysis of miRNA expression in normal thyroid tissue and in major types of thyroid tumours revealed that the majority of known miRNAs were expressed in normal thyroid tissues, whereas in thyroid neoplasms 32% of miRNAs were found to be consistently upregulated, and 38% were downregulated with more than a 2-fold change as compared to normal tissue (39). The most highly upregulated miRNAs in PDTC were miR-187, -221, -129, -222, -146b, -339, -183 (39).

The rearrangement involving the anaplastic lymphoma kinase (*ALK*) gene (40) and the striatin (*STRN*) gene (STRN/ALK) leads to constitutive activation of ALK kinase via dimerization mediated by the coiled-coil domain of STRN and to a kinase-dependent, thyroid-stimulating hormone-independent proliferation of thyroid cells. Expression of *STRN/ALK* transforms cells *in vitro* and induces tumour formation in nude mice. Kelly et al. (41) reported this gene arrangement in 9% of PDTC and demonstrated that *STRN/ALK* gene fusion occurs in a subset of patients with aggressive types of thyroid cancer, providing initial evidence that ALK inhibitors may represent a therapeutic option in these cases (41).

Other molecular alterations include downregulation of genes responsible for specialized thyroid function and in cell adhesion, upregulation of genes involved in motility and cell–cell interaction, and different patterns of deregulation of the expression of genes that encode cytokines and other proteins involved in inflammation and immune response. Although the list of specific deregulated genes varies substantially between different studies, a number of genes have been consistently found to be deregulated at the mRNA level: *MET*, *TPO*, *TIMP1*, *DPP4, LGALS3* and *KRT19* (42-46).

UNDIFFERENTIATED (ANAPLASTIC) CARCINOMA

For a thorough and extremely competent review on the macroscopic and histologic aspects of these tumours please see the respective AFIP chapter (8). A typical case of UTC is illustrated in Figure 2A,B. The immunohistochemical and molecular data on the whole group are summarized in Table 2.

Undifferentiated thyroid carcinoma (UTC) corresponds to the most aggressive form of thyroid cancer, being the final step of the progression of thyroid epithelial neoplasms. The concept of stepwise progression from a pre-existing well-differentiated thyroid carcinoma to poorly differentiated and undifferentiated carcinoma is supported by clinical, epidemiologic, pathologic and molecular evidence (studies of loss of heterozygosity, comparative genomic hybridization, genetic and epigenetic alterations), although anaplastic carcinoma may apparently also arise *de novo* (7, 47, 48).

Thyroid tumours are part of the minority of those human cancer types that do not follow the classical Vogelstein model, in which mutational inactivation of *TP53* is a crucial step in the first steps of progression (from adenoma

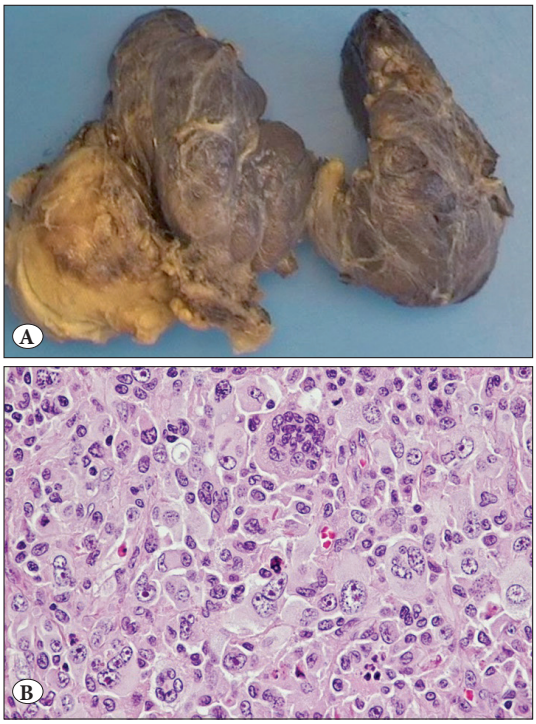


Figure 2: Undifferentiated Thyroid Carcinoma: A) Large tumour that grows beyond the thyroid limits, **B)** The neoplastic cells are mitotically active, atypical and pleomorphic displaying epithelioid to spindle cell morphology and co-existing with giant cells (H&E, 400x).

to carcinoma) (49). *TP53* gene inactivation seems to play a major role in the progression from differentiated to undifferentiated carcinoma, being a late event in the carcinogenic process and occurring together with a marked increase of cell proliferation (7). At variance with well-differentiated tumours in which TP53 gene mutations are rare, in UTC the loss of function mutations in different reports ranges from 50 to 80% (19, 27, 50-52). Many studies have shown that, when the same tumour contained well-differentiated and undifferentiated components, *TP53* mutation was restricted to the anaplastic foci (50, 53, 54). No clear prognostic significance has been attributed to the presence of *TP53* mutations in UTC.

The second most frequent genetic alteration in UTC is the recently described *TERT* promoter mutation. The -124 and -146 mutations in *TERT* promoter are detected in up to 50% of UTC; this percentage is higher to the percentage found in PDTC, and much higher than that reported in well-differentiated thyroid carcinomas (8, 55). The -124 mutation occurs more frequently, being the two mutations mutually exclusive. It was reported that -124 and -146 *TERT* promoter mutations coexist frequently with $BRAF^{V600E}$ mutation (33, 35, 36). In one report, Landa et al. (37) stated that *TERT* promoter mutations were collectively associated with *BRAF* and *RAS* mutations when UTC and PDTC were lumped together. Recently, Shi et al. (56) found that a-124 *TERT* promoter mutation in UTC is typically associated with older age of the patients and with distant metastasis, thus suggesting that this mutation plays an important role in the pathogenesis and aggressiveness of UTC.

The prevalence of *RAS* mutations in UTC ranges from 4 to 60% (13, 31, 57, 58). Activating point mutations typically affect codons 12, 13 and 61 of the *NRAS* gene. *In vitro RAS* activation leads to the rapid loss of differentiation markers, such as thyroglobulin, thyroid peroxidase, and thyrotropin receptor, and increased proliferation, but it is not sufficient to induce per se complete transformation of thyroid cells (59-61). Dedifferentiation is likely not driven by *RAS* mutations individually, but rather by the combined effect of multiple genetic alterations (62) including *TP53* and *TERT* promoter gene mutations

The $BRAF^{V600E}$ point mutation that constitutes about 98–99% of all BRAF mutations found in thyroid cancer, and it is present in up to 40% of UTC (25, 27, 63). Nikiforov et al. (47) reported that *BRAF* mutation was detected in areas of PTC and of PDTC or UTC coexisting in the same tumours, providing molecular evidence for stepwise progression from PTC to PDTC and UTC. The results on the record also suggest that constitutive activation of *BRAF* may predispose to such a progression, although it appears that this mutation represents an early event and additional genetic alterations (namely *TP53* mutation) are required to promote the process of dedifferentiation (47).

Molecular alterations are progressively accumulated in thyroid cancer during the dedifferentiation process, and this occurs particularly in those genes that encode effectors of the PI3K-AKT pathway. Among UTC, 5–25% of the tumours harbour mutations in *PIK3CA* (8), 5–15% *PTEN* mutations and 5–10% *AKT1* mutations (22, 27, 32, 64). Amplification of the *PIK3CA* genomic locus in 3q26.3 is found in about 40% of UTC suggesting that alteration of the PI3K-AKT pathway plays a pivotal role in the pathogenesis of UTC (55).

The *STRN/ALK* gene rearrangement has been identified in a subset of UTC that appears to develop from PTC. This gene rearrangement was also reported in one of 24 UTC coexisting with a follicular variant of PTC component (41). Murugan et al. (65) identified activating point mutations in *ALK* (encoding the tyrosine kinase domain of the protein) in two tumours from a series containing 18 UTC.

Recently, a critical role for miRNAs in carcinomas has emerged with increasing evidence showing that they may drive and potentiate tumourigenesis and neoplastic progression. Some miRNAs appear to be reduced in thyroid cancer, such as the let-7 family, but other miRNAs, such as the miR-200 and miR-30 families, are exclusively downregulated in UTC, indicating that its loss may play a role in the acquisition of more aggressive tumour characteristics (i.e., enhanced cell invasion and migration or dedifferentiation) (66, 67). On the other hand, miRNAs such as miR-146, miR-221, miR-222, and miR-17-92 are upregulated in UTC and in well-differentiated thyroid cancer, indicating that reinforced expression of these miRNAs appears to play a role in the maintenance of the oncogenic process (66).

The low-density lipoprotein receptor-related protein gene (LRP1B) originally isolated on the basis of homozygous deletions detected in human lung cancer cell lines (68) is among the top 10 most significantly deleted genes across 3312 human cancer specimens (69). LRP1B localizes at 2q21, a susceptibility locus for familial non-medullary thyroid cancer and encodes for a member of the endocytic low-density lipoprotein receptor superfamily (68). Our group reported that the LRP1B expression level in UTCs was significantly lower than in differentiated thyroid cancers, and that such reduced expression was due to frameshift mutation and genomic loss of *LRP1B* gene (68). Moreover,

UTC showed frequent methylation of the promoter region of the gene leading the loss of the expression of LRP1B in more than 80% of UTCs (68).

Among the proteins whose expression is altered in UTC, some appear as promising novel therapeutic targets namely Aurora kinases and transferrin receptor. In UTC, Aurora kinases are often found overexpressed (70). Aurora kinases are serine/threonine kinases that play an essential role in cell division. Their aberrant expression and/or function induce severe mitotic abnormalities resulting in either cell death or aneuploidy. Baldini et al. (71) reported the *in vitro* efficacy of Aurora kinase inhibitors (MLN8237 inhibitor for Aurora-A and AZD1152 inhibitor for Aurora-B) in restraining cell growth and survival of four human UTC cell lines (CAL-62, BHT-101, 8305C, 8505C). Baldini showed furthermore that Aurora A inhibition appears to be more effective than that of Aurora B in UTC cell lines (71). These data indicate that inhibitors for overexpressed proteins, such as Aurora kinases, alone or in combination with other drugs, including microtubule inhibitors, display an anticancer effect in preclinical models of UTCs suggesting that this approach may be used as an alternative therapeutic strategy for patients with UTC (72, 73).

The TfR1/CD71 is a cell membrane glycoprotein involved in iron homeostasis and cell growth. Parenti et al. (74) reported immunohistochemical data demonstrating the overexpression of TfR1/CD71 in UTC and discuss the possibility of targeting TfR1/CD71 by monoclonal or recombinant antibodies or transferrin-gallium-TfR1/CD71 molecular complexes or even small interfering RNAs (siRNAs) (74).

Finally, aberrant methylation of gene promoter regions and histone modifications can alter the function of tumour suppressor genes and thus contribute to activation of signalling pathways, such as PI3K–AKT and MAPK cascades. Changes in the epigenetic regulation of oncogenic steps might also lead to downregulation of thyroid-specific genes during tumour progression and dedifferentiation (75).

SMALL CELL THYROID CARCINOMAS

In thyroid, the small cell phenotype has been observed mainly in poorly differentiated carcinoma and lymphoma (76). Other primary tumours that may exhibit small cell features include medullary thyroid carcinoma, undifferentiated carcinoma, squamous cell carcinoma, CASTLE, primary extra-skeletal Ewing family tumours (PEEFTs) (76-79) as well as other rare flowers such as neuroblastoma (80) and basaloid neoplasm with solid cell nest features (81). Until now, the rare reports of primary neuroendocrine and non-neuroendocrine small cell carcinomas of the thyroid (82, 83) have not provided enough evidence to support the recognition of a primary small cell carcinoma of the thyroid as an entity.

In 2011, our group reported the first case of carcinoma of the thyroid with Ewing family tumour elements (CEFTE) (9). CEFTE is a unique small cell epithelial tumour that showed neither C cell nor follicular cell differentiation. It occurs in young patients and presents as large lobulated thyroid nodules. Histologically, CEFTE are unencapsulated, predominantly expansive neoplasms, displaying vascular invasion and growing in a nested pattern consisting of well-defined, variable sized insulae with central necrosis, as well as trabeculae and solid areas (Figure 3A,B). The cells are small, uniform, with regular and fairly round nuclei disclosing fine chromatin and variable nucleoli. The

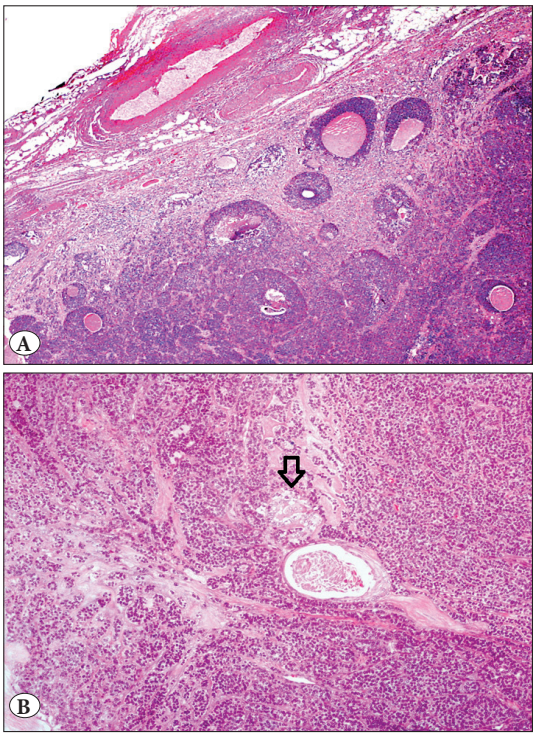


Figure 3: Histological aspect of carcinoma of the thyroid with Ewing family tumour elements (CEFTE): A) Nested pattern area with foci of necrosis (H&E, 20x), **B)** The majority of the neoplastic cells are small, there are foci of squamous differentiation and some follicles composed by cells with papillary thyroid carcinoma-type nuclei (arrow) (H&E, 100x).

cytoplasm is scant with ill-defined boundaries. Additionally, epidermoid-like areas and co-existing follicular foci with papillary-like nuclear features may be observed (9). Mitotic activity is variable.

The neoplastic cells of CEFTE diffusely express cytokeratins, p63, E-cadherin and CD99 in the absence of vimentin expression (9-11). In Table 3 we summarized the immunohistochemical features of CEFTE. CEFTE should be distinguished from basaloid tumor with solid cell nests features that is a PTC related tumor composed by small cells that express p63, cytokeratin 5 and galectin3 in the absence of CD5 and CD99 expression (81).

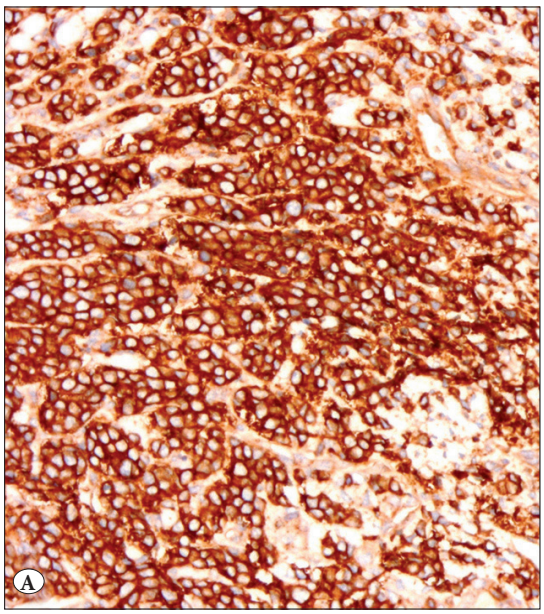
CEFTE discloses the EWSR1-FLI1 rearrangement typical of PEEFTs (9-11) (Figure 4A,B). We do not know if CEFTE should be considered as a type of PEEFT with extensive carcinomatous differentiation, or true small cell non-neuroendocrine carcinoma exhibiting the *EWSR1-FLI1* rearrangement. As in other locations, the diagnosis of thyroid PEEFT relies on the detection of the typical *EWSR1-FLI1* rearrangement (84). The *EWSR1* rearrangements with other partners have been reported in other non-PEEFT tumours (85,86).

It is worthwhile stressing the apparent good prognosis of these tumours, despite their poorly differentiated appearance, vascular invasiveness and necrotic foci.

The etiopathogenesis of such small cell carcinomas of the thyroid remains unknown: do they derive from "dedifferentiated" PTC cells that have acquired the *EWSR1-FLI1* rearrangement and entirely lost thyroid differentiation

Table III: Immunohistochemical features of carcinoma of the thyroid with Ewing family tumour elements (CEFTE)

Antibody	Expression
Pan-cytokeratins	Present
Cytokeratin 5	Absent
Cytokeratin19	Present
Vimentin	Absent
TTF1	Absent
Thyroglobulin	Absent
Calcitonin	Absent
p63	Present
Chromogranin	Variable
Synaptophysin	Variable
CD99	Present
Galectin3	Absent
Ki67 labeling index	Variable



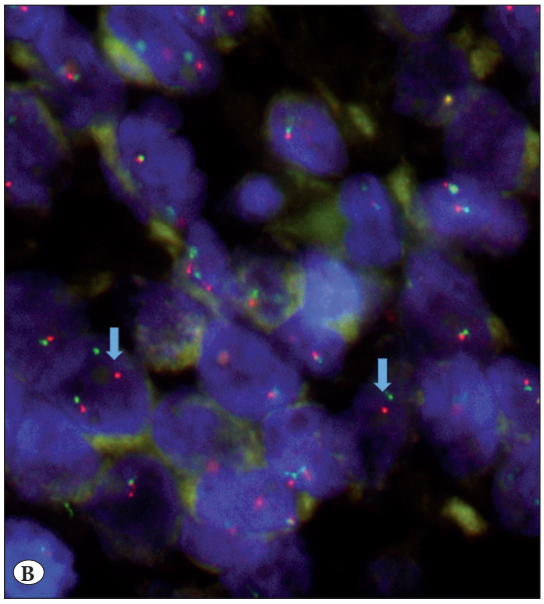


Figure 4: CD99 expression and EWSR1/FLI1 rearrangement in a case of carcinoma of the thyroid with Ewing family tumour elements (CEFTE): A) CD99 diffuse and membrane expression in the small cells (200x), **B)** EWSR1/FLI1 rearrangement detected by FISH dual color probe in the same case (break apart signs – arrow).

(negativity for TTF-1 and thyroglobulin), or do they originate from thymic/branchial pouch remnants such as the main cells of solid cell nests? (10).

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CONFLICT OF INTEREST

Authors declared that they have no conflict of interest.

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7.1.2 Paper II - Osteopontin-c mediates the upregulation of androgen responsive genes in LNCaP cells through PI3K/Akt and androgen receptor signalling

Osteopontin-c mediates the upregulation of androgen responsive genes in LNCaP cells through PI3K/Akt and androgen receptor signaling

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Abstract. Androgen receptor (AR) signaling is a key pathway modulating prostate cancer (PCa) progression. Several steps in this pathway have been investigated in order to propose novel treatment strategies for advanced PCa. Total osteopontin (OPN) has been described as a biomarker for PCa, in addition to its role in activating the progression of this tumor. Based on the known effects of the OPNc splice variant on PCa progression, the present study investigated whether this isoform can also modulate AR signaling. In order to test this, an *in vitro* model was used in which LNCaP cells were cultured in the presence of conditioned medium (CM) secreted by PCa cells overexpressing OPNc (OPNc-CM). The activation of AR signaling was evaluated by measuring the expression levels of AR-responsive genes (ARGs) using quantitative polymerase chain reaction and specific oligonucleotides. The data demonstrated that all nine tested ARGs (*Fgf8*, *TMPRSS2*, *Greb1*, *Cdk2*, *Ndrg1*, *Cdk1*, *Pmepa1*, *Psa* and *Ar*) are significantly upregulated in response to OPNc-CM compared with LNCaP cells cultured in CM secreted by control cells transfected with empty expression vector. The specific involvement of OPNc was demonstrated by depleting OPNc from OPNc-CM using an anti-OPNc neutralizing antibody. In addition, by using a phosphoinositide 3-kinase (PI3K)-specific inhibitor and AR antagonists, such as flutamide and bicalutamide, it was also observed that upregulation of ARGs in response to OPNc-CM involves PI3K signaling and depends on the AR. In conclusion, these data indicated that OPNc is able to activate AR signaling through the PI3K pathway and the AR. These data further corroborate our previous data, revealing the OPNc splice variant to be a key molecule that is able to modulate key signaling pathways involved in PCa progression.

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Introduction

Osteopontin (OPN) is a matricellular glyco-phosphoprotein that is overexpressed in several tumor types (1). In prostate cancer (PCa) samples, OPN is upregulated and mediates tumor progression (2,3). Moreover, high circulating OPN levels have been found in PCa patients, thus highlighting a putative biomarker role for OPN in PCa. The OPN transcript can occur as three distinct splice variants, OPNa, OPNb and OPNc (4), with tissue- and tumor-specific roles (5). Nonetheless, the majority of studies have explored the function of the full-length OPN in PCa (6-9). Notably, we have previously demonstrated that the overexpression of OPNc, and to a lesser extent OPNb, promotes PCa progression (10). Indeed, OPNc upregulation in PCa cells, which correlates to the Gleason score, induces PCa cell proliferation, migration, invasion, metastasis and tumor formation *in vivo*, mainly mediated by the PI3K pathway. Overall, our studies have shed light on the potential use of OPNc as a diagnostic and prognostic biomarker for PCa (11).

Disruption of androgen-mediated differentiation has been strongly linked to PCa development. Androgens, which bind to androgen receptors (AR) to elicit their cellular effects, are the primary sex hormones required for normal development, maintenance and differentiation of the male phenotype. AR-regulated genomic events modulate cell differentiation and the development of tissues and organs (12). Furthermore, AR signaling has been indicated as a key step for PCa progression (13), in which circumstance there is crosstalk with multifunctional growth factor signaling pathways, such as EGF, FGF, IGF, TGF-β and VEGF (14), as well as with the PI3K/Akt/mTOR pathway (15). In addition, it has been proposed that castration-resistant PCa evolution may be the result of increased growth factor signaling activity associated with intratumoral testosterone production (16,17).

Despite the key importance of AR signaling on PCa progression, to date, there have been no studies demonstrating

the putative correlations among total OPN or its splice variants and AR signaling. The exception to this is a single study, which reported that upregulated *Fgf-8*, an androgen target gene, induces total OPN expression in PCa cells (18).

The present study describes an *in vitro* model in which LNCaP androgen-responsive PCa cells are used to investigate the differential modulation of AR target genes by the conditioned medium (CM) secreted by PCa cells overexpressing OPNc (OPNc-CM).

Materials and methods

Cell culture. The LNCaP cell line was used as an *in vitro* model to examine whether the AR pathway modulated by OPNc in PCa cells. The LNCaP cell line was obtained from the American Type Culture Collection (Rockville, MD, USA) and maintained in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; Invitrogen Life Technologies, Carlsbad, CA, USA), in the presence of 100 U/ml penicillin and 100 μg/ml streptomycin, at 37˚C in a 5% CO_2 humidified incubator. The cells were maintained in medium containing charcoal/dextran-stripped FBS (CCS; Invitrogen Life Technologies) for three days prior to assaying the modulatory effect of OPNc-overexpressing secreted CM on LNCaP cell AR signaling.

OPNc plasmid constructs, transfection and preparation of CM. In order to prepare the OPNc-CM, OPNc overexpression vector, which was kindly donated by Dr George Weber (Cincinnati University, Cincinnati, OH, USA), was used for transfections into a PC-3 prostate tumor cell line. The transfections were performed using Lipofectamine™ 2000, following the manufacturer's instructions (Invitrogen Life Technologies). Cell clones stably overexpressing OPNc and empty vector (EV) control clones were selected using G418 at 800 μg/ml. Data from our previous study demonstrated that PC-3 stably transfected cells contain high levels of the protein and RNA transcript of OPNc in relation to their endogenous levels in EV-transfected cells (10). In order to prepare the CM secreted from OPNc-overexpressing cells and those expressing EV, cell number was normalized by plating PC-3 cells at the same cell density ($5x10^5$ cells/well). Subsequent to reaching 80% cell confluence, the cells were washed twice with phosphate-buffered saline and cultured with RPMI in serum-free conditions for 48 h. Collected CM was clarified by centrifugation at 1,200 x g for 5 min. All assays were performed using freshly prepared CM. CM produced by OPNc-overexpressing cells or those transfected with EV controls, termed OPNc-CM and EV-CM, respectively, were used for the LNCaP assays over 24 h.

LNCaP assays and AR signaling analysis. The LNCaP cells were plated in 2.0 ml RPMI without antibiotics at a density of $1.5x10^5$ cells/well, and maintained in medium containing CCS (Invitrogen) for three days prior to treatment with OPNc-CM or EV-CM, containing either anti-OPNc neutralizing antibody, LY294002, flutamide and bicalutamide, individually or in distinct combinations. LY294002, a PI3K inhibitor, was obtained from Cell Signaling Technology Inc. (Danvers, MA, USA). The LNCaP cells were cultured and treated with 50 mM LY294002. For OPNc depletion in OPNc-CM, 4 mg/ml of an anti-OPNc antibody (Gallus Immunotech, Cary, NC, USA) was used. This antibody was produced by immunizing a chicken with a peptide representing the splice junction of OPNc (Ac-SEEKQNAVSCCOOH). Specific binding to OPNc has been demonstrated by the manufacturers (Gallus Immunotech), and we have previously demonstrated that this antibody blocks PC3 cell proliferation in response to OPNc-overexpression (10). OPNc-CM was pre-incubated with anti-OPNc antibody for 2 h prior to LNCaP cell treatment. Assays using AR antagonists were performed using OPNc-CM containing 100 nM flutamide or 10 μM bicalutamide (Sigma-Aldrich). The LNCaP cells were allowed to grow for 24 h following the treatments, and then harvested for the analysis of gene expression. The mRNA expression levels of the androgen-responsive genes (ARGs), *Ar*, *Psa*, *Tmprss2*, *Ndrg1*, *Greb1*, *Fgf8*, *Cdk1*, *Cdk2* and *Pmepa1*, were analyzed using quantitative reverse transcription polymerase chain reaction (RT-qPCR).

Total RNA isolation and RT. LNCaP total RNA was purified using the RNeasy Mini kit, using RNase-free DNase (Qiagen, Hilden, Germany) following the RNA purification process. Total RNA (1 μg) was reverse-transcribed into cDNA using a Superscript II First-Strand Synthesis System for RT-PCR (Invitrogen). The resulting cDNA was quantified using a NanoDrop™ 1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA).

qPCR. qPCR was performed using a CFX96 Real-Time System with a C1000 Thermal Cycler (Bio-Rad, Hercules, CA, USA), and SYBR Green (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. The oligonucleotide primers used for the qPCR are listed in Table I. The expression levels of *Ar*, *Psa*, *Tmprss2*, *Ndrg1*, *Greb1*, *Fgf8*, *Cdk1*, *Cdk2* and *Pmepa1* were normalized based on the reference gene (18S rRNA), using the ΔΔCT relative quantification method. Conditions for PCR amplification were as follows: 50˚C for 2 min and 94˚C for 5 min, followed by 40 cycles at 94˚C for 30 sec, 50˚C for 30 sec and 72˚C for 45 sec, and a final extension at 72˚C for 15 min. To evaluate the specificity of the PCR products, a melting curve analysis was performed after each reaction.

Statistical analyses. All the statistical analyses were performed using SPSS software version 18.0 (SPSS, Inc., Chicago, IL, USA). Data were analyzed by comparison using a two-tailed t-test, and $P<0.05$ was considered to indicate a statistically significant difference.

Results and Discussion

An improved understanding of the molecular mechanisms triggering AR signaling in PCa cells strongly relies on dissecting gene products and factors able to stimulate this hormonal pathway. Moreover, novel therapeutic strategies against PCa, mainly in recurrent disease, may attempt to target AR pathway elements or its stimulatory mechanisms (17). We previously demonstrated that the OPNc splicing isoform stimulates several PCa tumor progression features, including

Table I. Oligonucleotide primers used for analysis of RT-qPCR expression of androgen receptor-responsive genes.

Gene	Accession number	Primer (5'-3')
Pmepa1	NC_000020.11	F: CATGATCCCCGAG CTGCT R: TGATCTGAACAAACTCCAGCTCC
TMPRSS2	NC_000021.9	F: CTGGTGGCTGATAGGGGATA R: GGACAAGGGGTTAGGGAGAG
Ndrg1	NC_000008.11	F: CGAGACTTTACATGGCTCTG R: GCATTGATGAACAGGTGCAG
Greb1	NC_000002.12	F: AAGGAGGGCTGGAAACAAAT R: CATTGTGGCCATTGTCATCT
Psa	NC_000019.10	F: TGCATCAGGAACAAAAGCGTGA R:CCTGAGGCGTAGCAGGTGGTCCCCAG
Ar	NC_000023.11	F: GGTGAG CAGAGTGCCCTATC R: GAAGACCTTGCAGCTTCCAC
Fgf8	NC_000010.11	F: CAACTCTACAGCCGCACCAGC R: TGCTCTTGGCGATCAGCTTC
Cdk1	NC_000010.11	F: AAGTGAAGAGGAAGGGGTTCC R: CCAAAAGCTCTGGCAAGGCC
Cdk2	NC_000012.12	F: ATGGGTGTAAGTACGAACAGG R: TTCTGCCATTCTCATCGG
18S	NT_167214.1	F: AACCCGTTGAACCCCATT R: CCATCCAATCGGTAGTAGCG

F, forward; R, reverse.

cell proliferation, migration, invasion, metastatic potential and tumor formation *in vivo* (10). Although other studies have shown that total OPN stimulates LNCaP cell proliferation in the presence of EGF (19), to date, information about the effect of distinct OPN splice variants on AR pathway modulation in PCa is lacking. We have previously shown that the majority of the OPNc-mediated PCa features are specifically modulated by OPNc-CM (10). In addition, we have demonstrated that OPNc-CM secreted by PC3 cells differentially modulates several cancer-related genes (20).

The present study used OPNc-CM to investigate the modulation of AR signaling, by evaluating the expression patterns of ARGs in LNCaP androgen-responsive cells. OPNc-CM, but not EV-CM, significantly increased the expression of all nine ARGs tested (Fig. 1). These data support our previous findings that OPNc stimulates several aspects of PCa progression (10), possibly through an AR signaling-mediated pathway. All tested ARGs have been described with regard to the modulation of PCa growth and progression (21); the *Fgf8* (22), *Cdk1* (23), *Cdk2* (24) and *Greb11* (25) gene products are classically involved in prostate cell growth and proliferation. Although *Psa* has been classically described as an oncogene in PCa, promoting tumor progression and metastasis, its function as a tumor suppressor molecule has been also documented (23). *NDRG1-ERG* fusions, which encode a chimeric protein, are also regulated by androgens and correspond to one of the recurrent erythroblast transformation-specific rearrangements observed in PCa. Presumably, *Ndrg1* promotes angiogenesis, metastasis and drug resistance (26). *Tmprss2*, which is another component of typical androgen-regulated PCa translocations, is expressed in PCa cells and contributes to prostate tumorigenesis (27,28). By contrast, the *Pmepa1* gene, although a direct target of the AR, has been described as negatively regulating prostate epithelium cell growth, in addition to the AR protein levels in a range of cell culture models (29,30).

In order to investigate the specificity of the effect of OPNc on the upregulation of tested ARGs, LNCaP cells were cultured with OPNc-CM pre-treated with an anti-OPNc polyclonal neutralizing antibody. The expression of seven out of nine of the ARGs (*Fgf8*, *Tmprss2*, *Greb1*, *Cdk2*, *Ndrg1*, *Cdk1* and *Pmepa1*) was not increased when OPNc activity was abrogated by this anti-OPNc antibody. These data indicated the specific effect of OPNc on inducing the upregulation of these seven ARGs in response to OPNc-CM (Fig. 1A-G). Conversely, *AR* transcript upregulation was sustained regardless of the OPNc activity, therefore suggesting that secreted factors contained in OPNc-CM, other than OPNc, could mainly contribute to *AR* transcript expression in PCa cells. Lastly, abrogation of OPNc activity further stimulated *PSA* expression in the LNCaP cells (Fig. 1I), thus indicating that secreted OPNc is a partial inhibitor of *PSA* expression in the PCa cell line. These results indicate that direct or indirect OPNc-mediated mechanisms, either in the OPNc-CM or within the LNCaP cells, could suppress PSA transcript expression. It has previously been demonstrated that during PCa progression, intracellular PSA levels are lower in the malignant rather than the normal prostatic epithelium, being further reduced in poorly-differentiated tumors, despite the high serum levels of PSA detected in

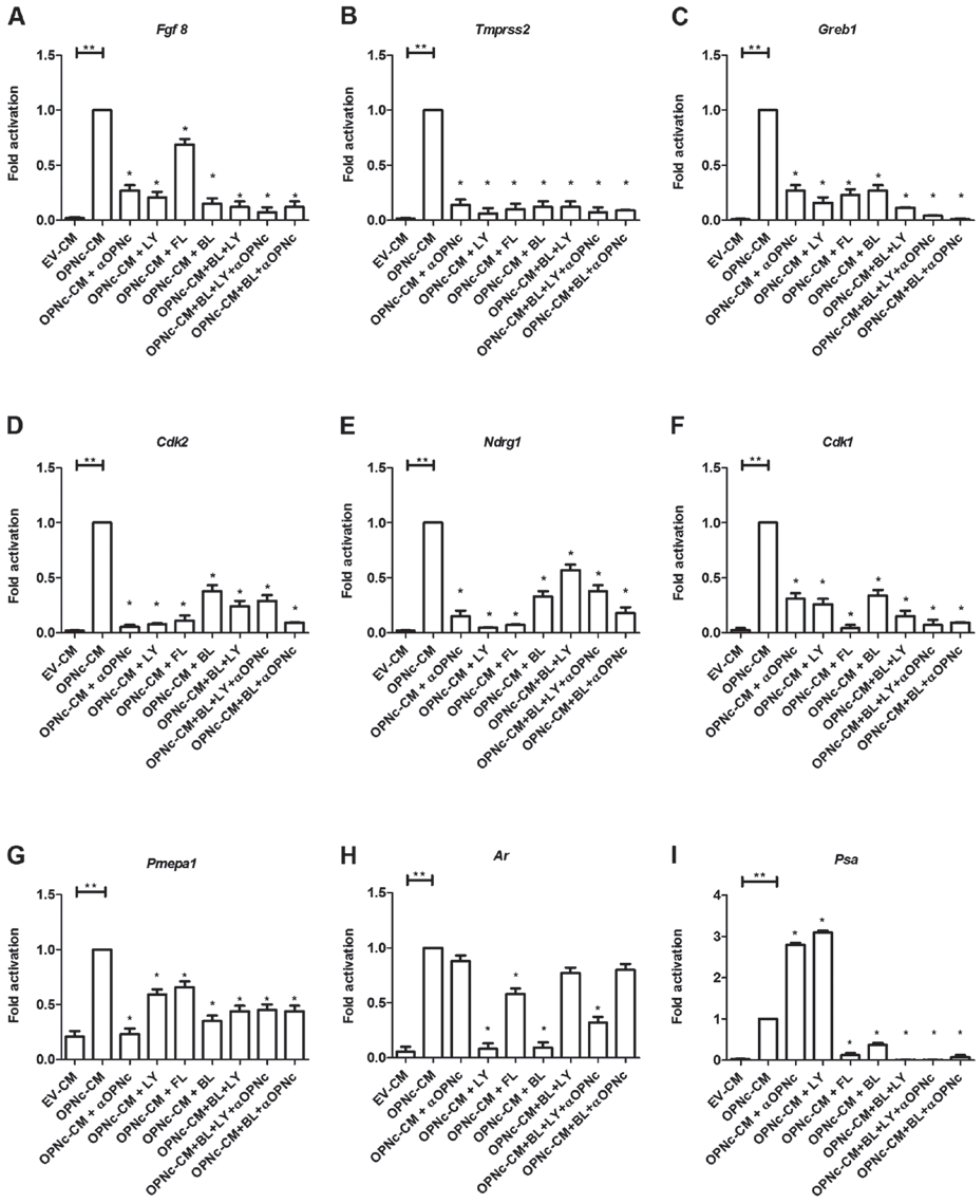

Figure 1. Conditioned medium (CM) secreted by PCa cells overexpressing OPNc (OPNc-CM) significantly activates the expression of AR responsive genes in LNCaP cells. Graphs showing relative RNA quantification of (A) *Fgf8*, (B) *Tmprss2*, (C) *Greb1*, (D) *Cdk2*, (E) *Ndrg1*, (F) *Cdk1*, (G) *Pmepa1*, (H) *Ar* and (I) *Psa*, in LNCaP cells cultivated with OPNc-CM compared with cells cultivated with the empty-vector CM (EV-CM), as described in the Materials and methods section. In order to test OPNc-specific effects, anti-OPNc polyclonal neutralizing antibody (α-OPNc), flutamide (FL), bicalutamide (BL) and PI3K inhibitor (LY), were used. 18S RNA was used as a constitutive gene in all these assays. Data are represented as the mean ± standard deviation. All experiments were biological replicates repeated a minimum of three times. *P<0.0001 vs. OPNc-CM cultivated cells. **P<0.0001 vs. EV-CM cultivated cells.

patients with PCa. Moreover, PSA can function as a tumor suppressor by inhibiting tumor angiogenesis in PCa cells (31). In fact, regardless of the widespread use of PSA as a PCa marker, it has been established that the modulation of PSA expression and the proliferation of PCa cells are independently regulated during the development and progression of the disease. It is notable that multiple factors have been indicated to be involved in the transcriptional transactivation of PSA; mainly AR, but also various growth factors and extracellular matrix proteins (32,33). We hypothesize that this could be the case for OPNc. Hence, it is possible that OPNc or growth factors secreted in response to the overexpression of this splice

variant could negatively modulate PSA expression in LNCaP cells, using androgen-dependent and/or independent mechanisms, in order to favor PCa progression.

The precise mechanisms responsible for the aberrant AR expression in PCa remain elusive (34). The data from our studies has shed light on the complexity of the phenomenon, indicating that not only secreted OPNc, but also other secreted factors in response to OPNc overexpression, positively modulate AR signaling in LNCaP cells. In this context, other studies have also described the stimulatory effects of extracellular and intracellular signaling molecules on AR-mediated transcription, such as heparin-binding EGF-like growth factor, activin A, Smad2 and angiotensin II receptor type 1 (35). Further studies should be performed to determine the factors produced in response to OPNc overexpression, as well as the molecular mechanisms these molecules can induce in order to modulate AR-mediated signaling in PCa cells.

Our previous studies recently demonstrated that OPNc modulates the PI3K pathway (10), as well as other key cancer pathways (20). As a consequence, we hypothesize that OPNc modulates the signaling of several growth factors in PCa cells, which in turn, induce ARG expression, exactly as previously reported for other oncoproteins, such as PLK1 (36), ETV1 (37) and ELK1 (38). It is possible that OPNc can also stimulate LNCaP endogenous androgen synthesis, similar to the role of interleukin-6 on the *de novo* synthesis of intracrine androgens (39). Indeed, our previous data have clearly shown that PI3K mediates several OPNc tumor progression features in PC3 cells overexpressing this splice variant (10). In the present study, it was demonstrated that the PI3K pathway was also involved with OPNc-mediated ARG expression in the LNCaP cells, with the exception of PSA, whose expression was not abrogated by the PI3K inhibitor LY294002 (Fig. 1). Altogether, these results shed light on the PI3K pathway as a key mechanism for OPNc-mediated effects on LNCaP cells (10,15). Upon further consideration of the crosstalk between the PI3K and AR pathways, we postulate that OPNc can modulate each pathway (15). In this context, we propose that the observed PSA upregulation, regardless of the PI3K pathway activity status, could be mainly due to the depletion of OPNc-activated signals as an inhibitor of PSA expression, similar to the experimental conditions in which the anti-OPNc antibody was used to abrogate OPNc activity in OPNc-CM. We finally propose that the tumor progression features modulated by OPNc in PCa cells may be derived, at least in part, from the PI3K-activated upregulation of ARGs.

Significantly, the present study demonstrated that AR mediates the OPNc-CM-activated upregulation of all ARGs tested, as the observed phenomena were significantly reversed when the LNCaP cells were pre-treated with the AR antagonists flutamide and bicalutamide (Fig. 1). The expression of the OPNc-CM-activated ARGs was also assessed in the LNCaP cells in the presence of LY294002 or bicalutamide. With the exception of AR, the expression of all other ARGs was significantly reduced. Also, the combined treatment with LY294002 and bicalutamide promoted a stronger effect on the inhibition of the OPNc-CM-mediated upregulation of the ARGs in the LNCaP cells, as compared to the individual bicalutamide or LY294002 treatments. Therefore, these data could provide support to strategies that could target the PI3K and AR signaling pathways as an efficient approach to inhibit PCa progression, as discussed in the study by Bitting and Armstrong (15).

In conclusion, to the best of our knowledge, the present study is the first to use PCa cells overexpressing OPNc and report that OPNc and/or other secreted factors are key elements modulating the AR signaling pathway. Briefly, the data indicate that OPNc-CM induces the expression of ARGs in LNCaP cells mainly through the activation of the PI3K and AR pathways; the latter being activated either by secreted OPNc-CM or LNCaP endogenously-produced AR ligands. This reinforces that these signaling pathways have key roles in mediating OPNc-related tumor progression features in PCa. Further studies should investigate the specific molecular pathways by which OPNc modulates the AR signaling and the secreted factors expressed in response to OPNc overexpression that could also contribute to AR signaling activation. Based on these results, therapeutic strategies trying to target OPNc and its downstream PI3K and AR pathways should also be considered in order to negatively modulate PCa progression.

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